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SYMPOSIA ISSUE

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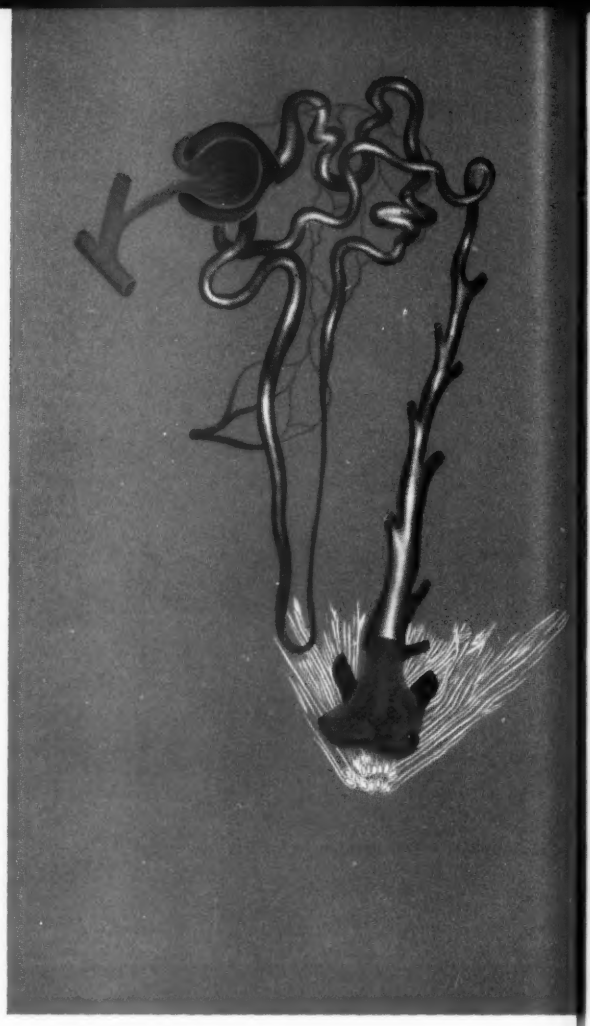

on the pathogenesis of pyelonephritis:

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The "exquisite sensitivity"[2] of the medulla to infection (as compared with the cortex), highlights the importance of obstruction to the urine flow in the pathogenesis of pyelonephritis. "There is good cause to support the belief that many, perhaps most, cases of human pyelonephritis are the result of infection which reaches the kidney from the lower urinary tract."[3]

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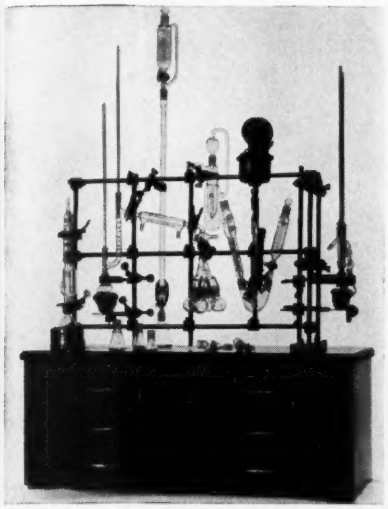

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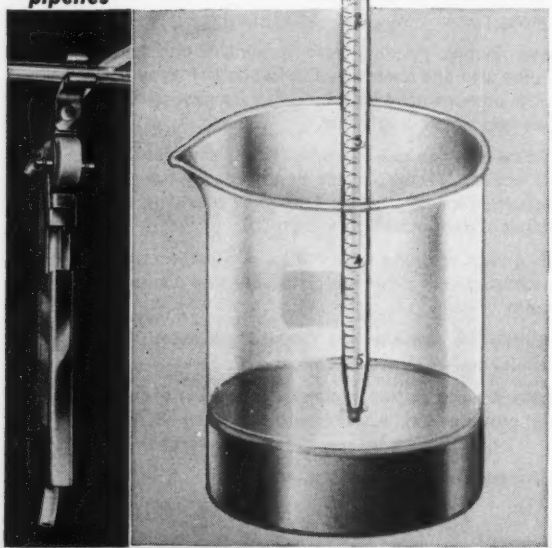

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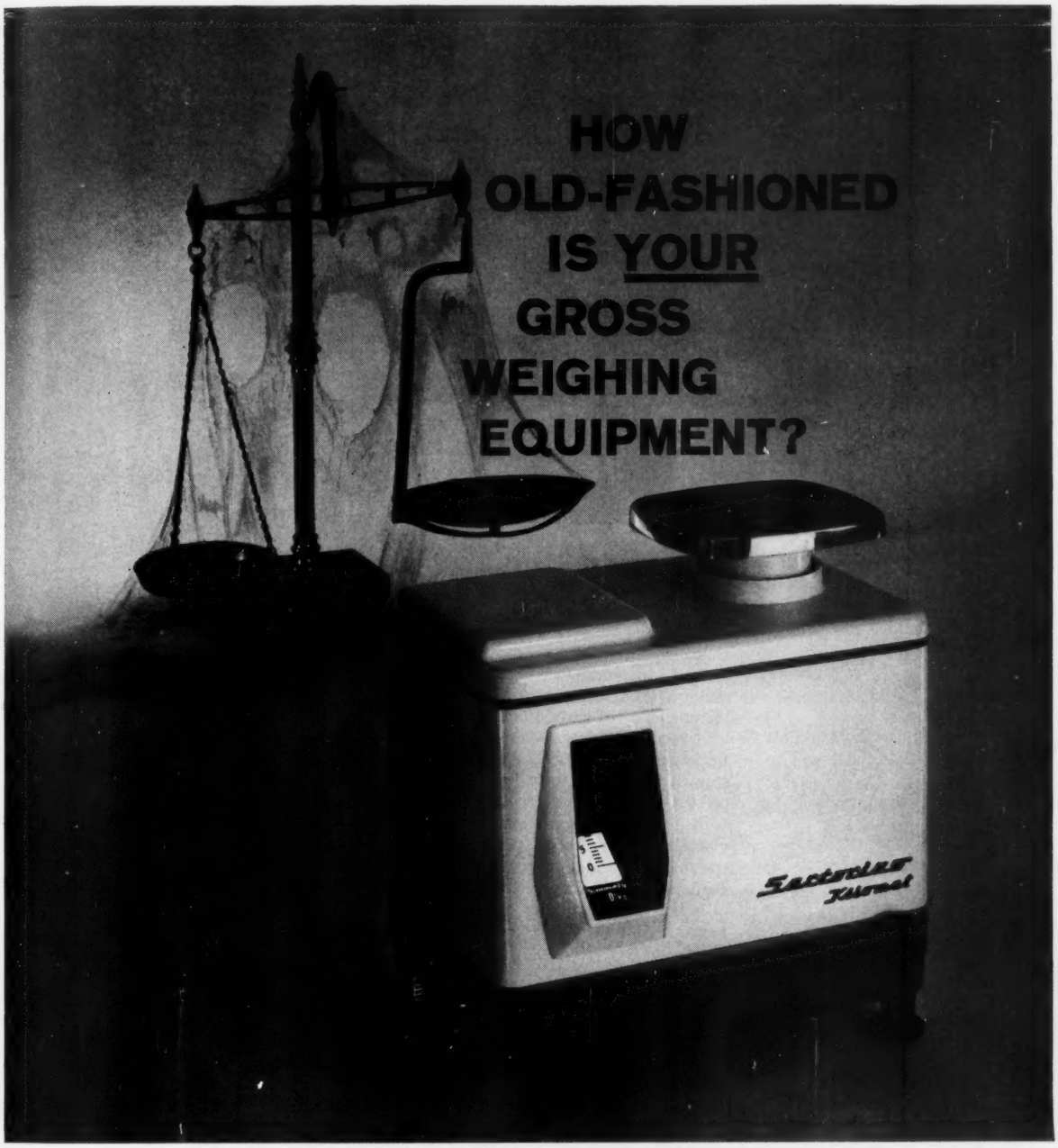

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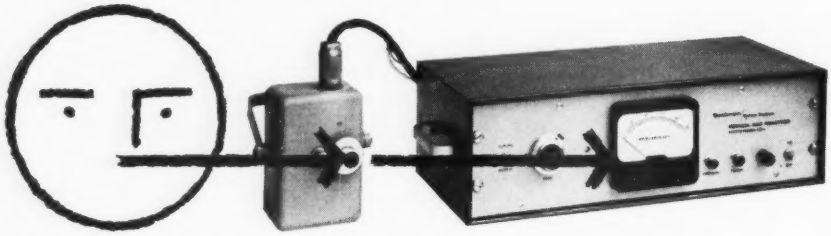

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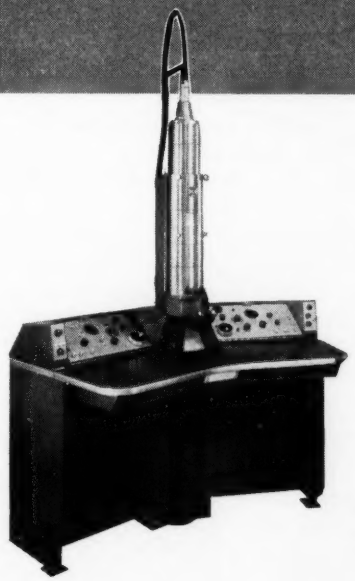

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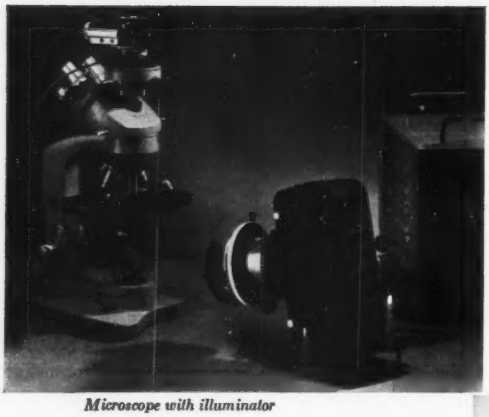

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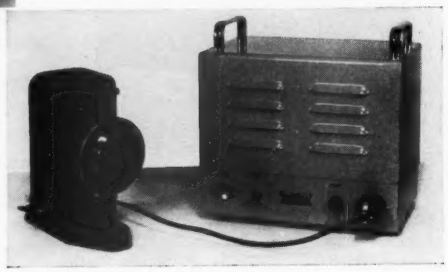

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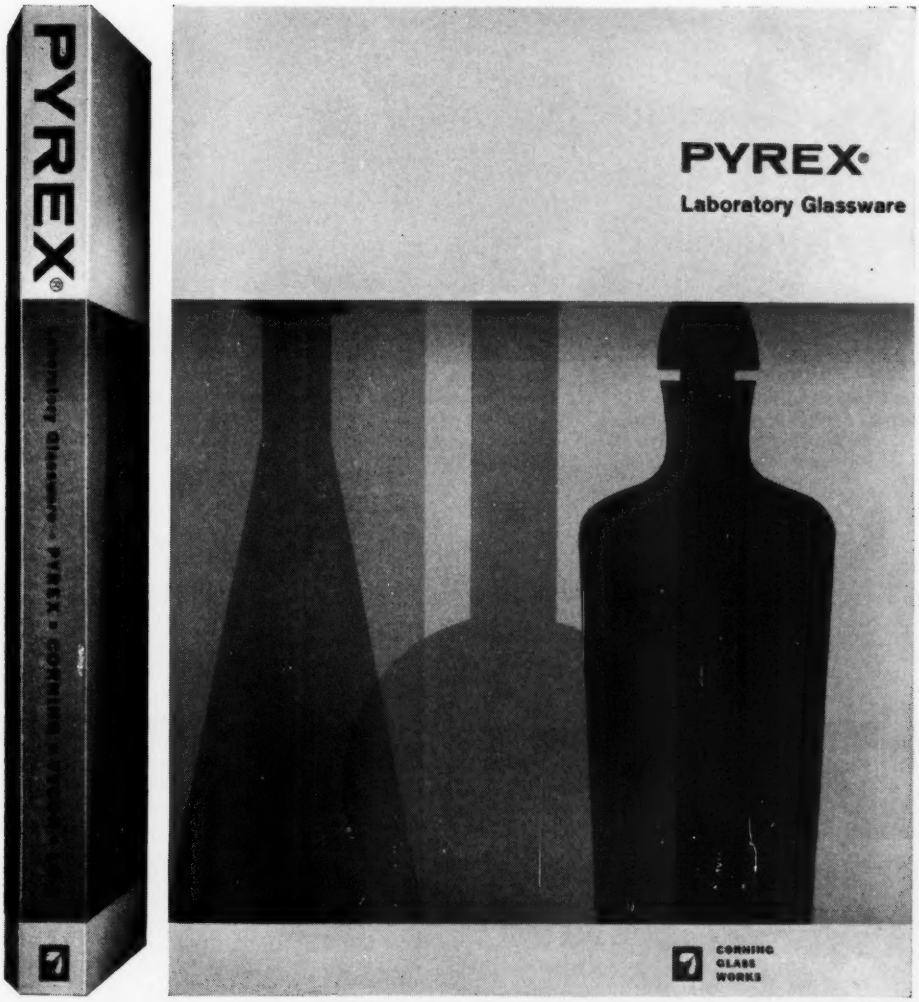

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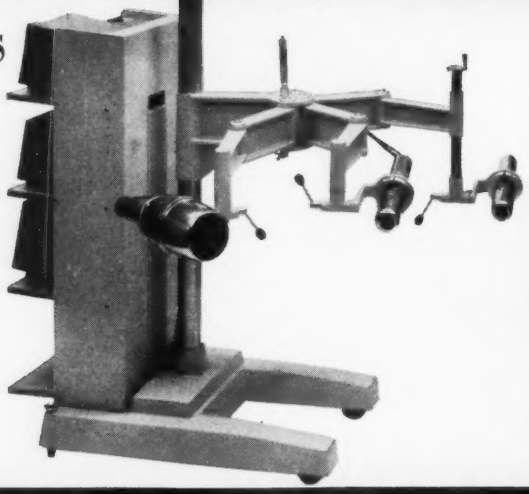

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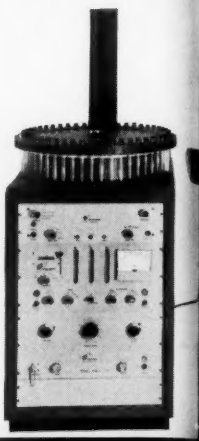

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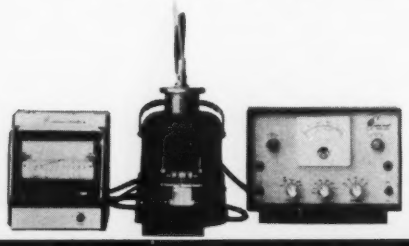

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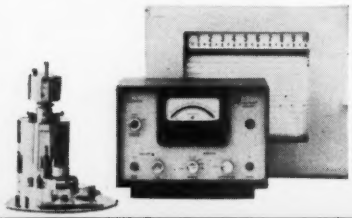
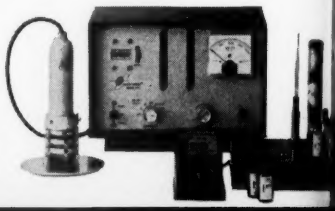


Model 6000 Dynacon Electrometer measures beta radiation in solid, liquid, or gaseous phase samples. It is ideal for use in continuous flow analysis.



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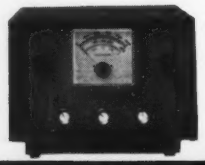

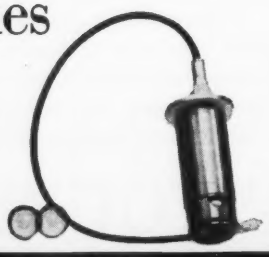

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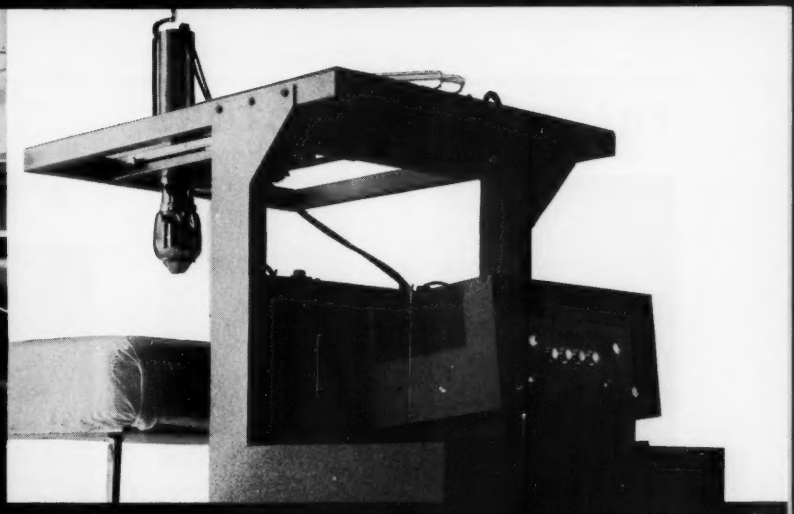
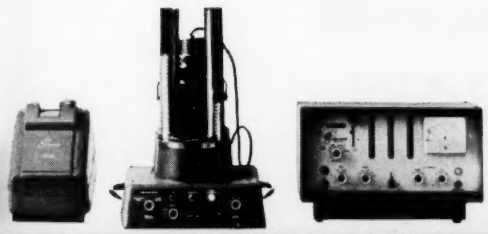
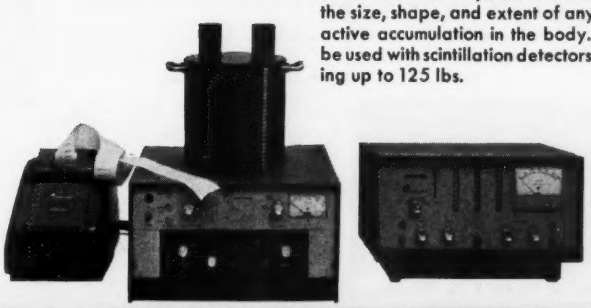


(Above) Model 1700A Isotope Scanner is an ideal clinical system for determining the size, shape, and extent of any radioactive accumulation in the body. It may be used with scintillation detectors weighing up to 125 lbs.



Model C110B Automatic Sample Changer accepts up to 35 solid beta emitting samples for automatic analysis. It may be used with window or windowless gas-flow detectors and end-window G-M Tubes.



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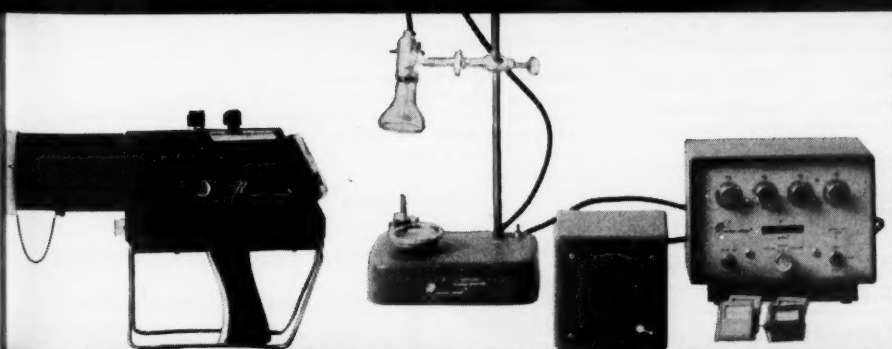

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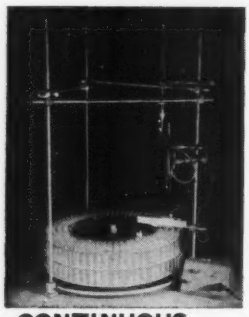

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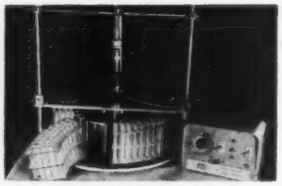
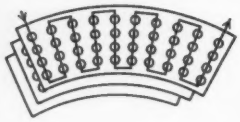


SECTIONAL . . . for processing during fraction collecting.



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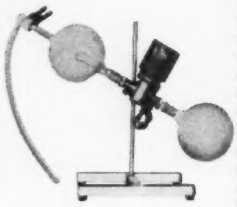

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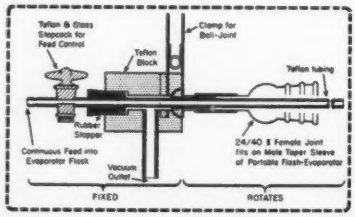

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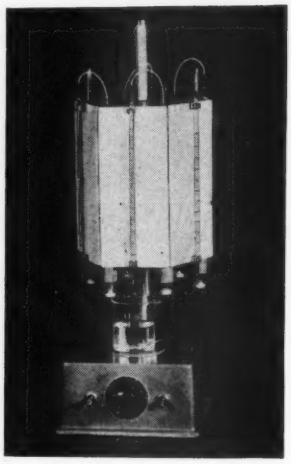

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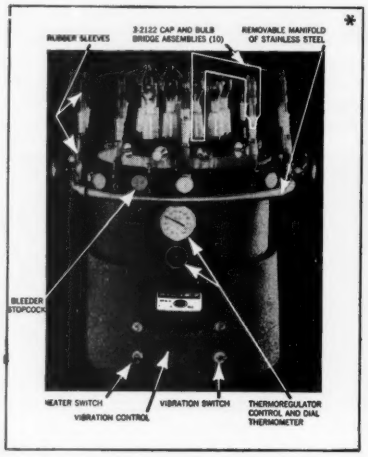

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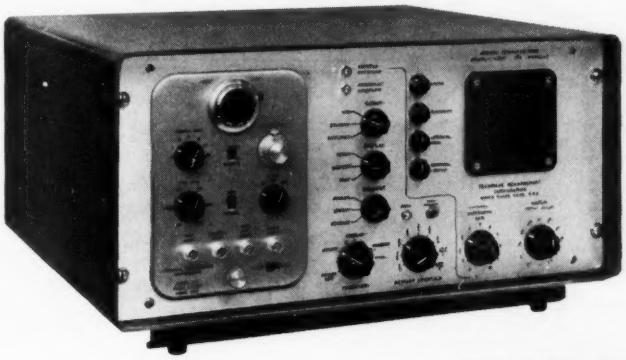

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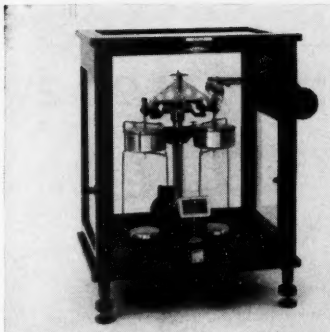

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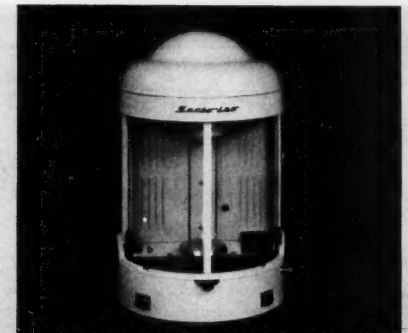

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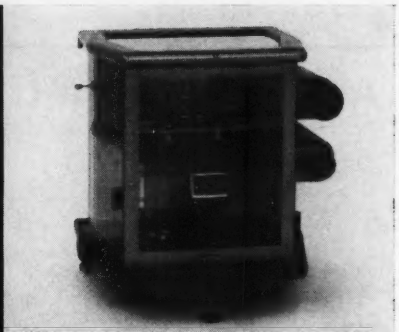

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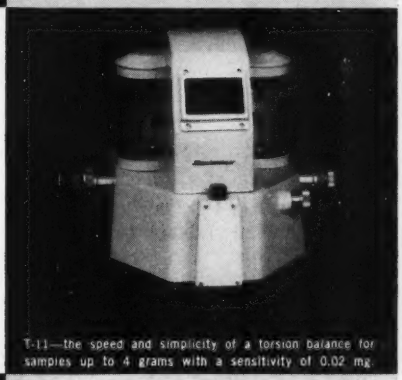

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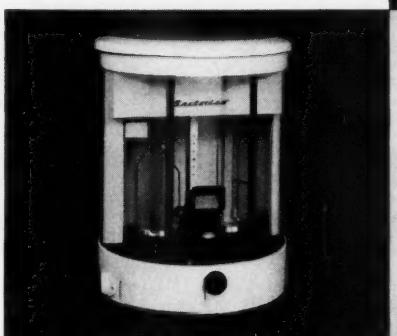

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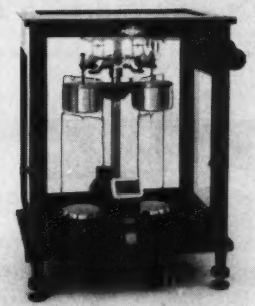

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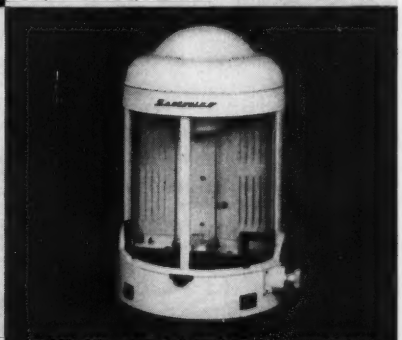

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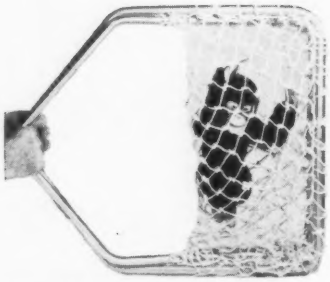

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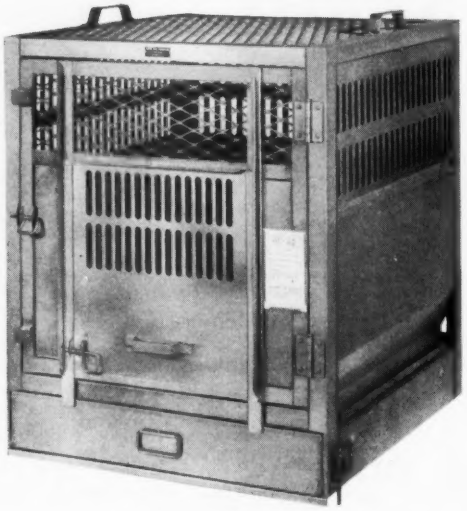

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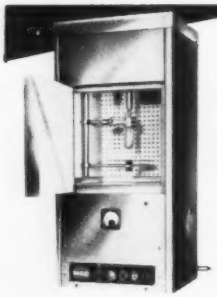

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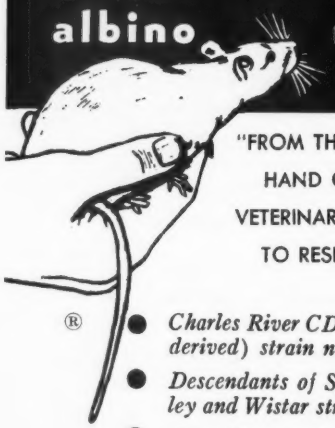

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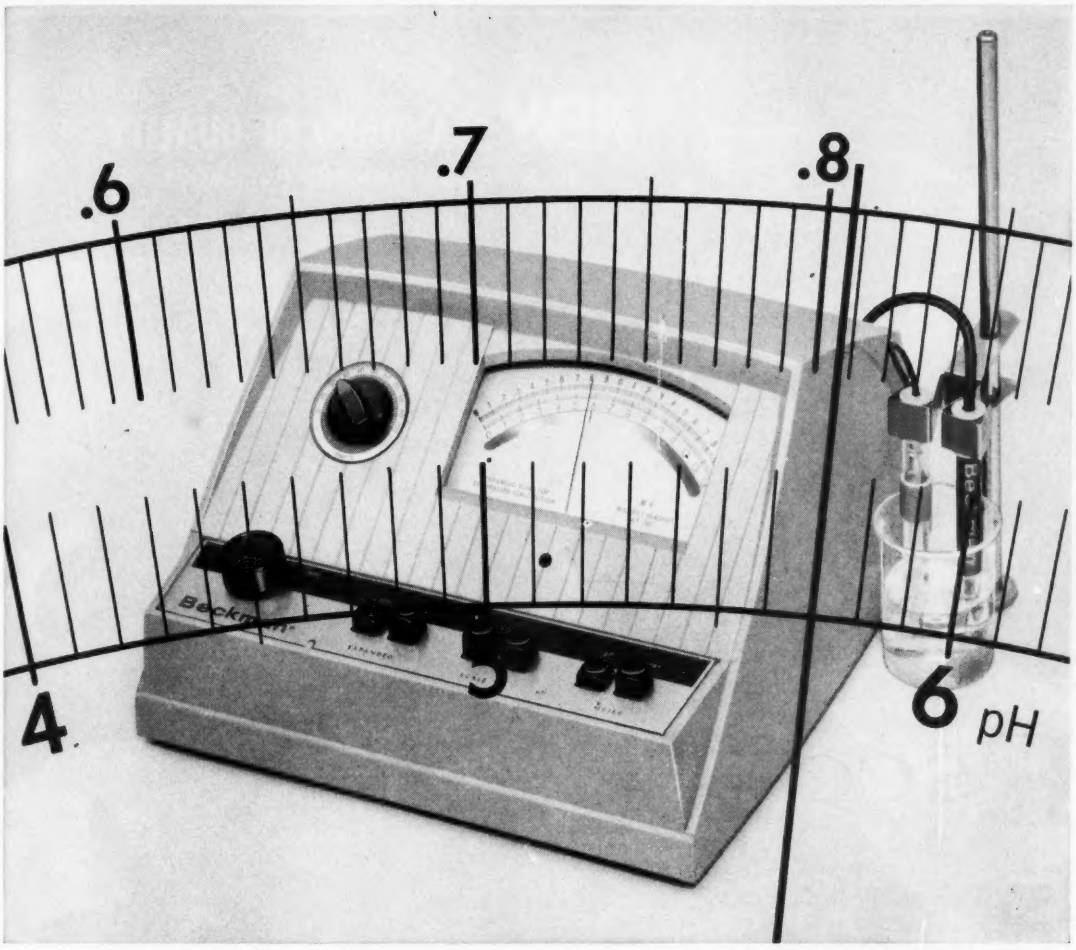

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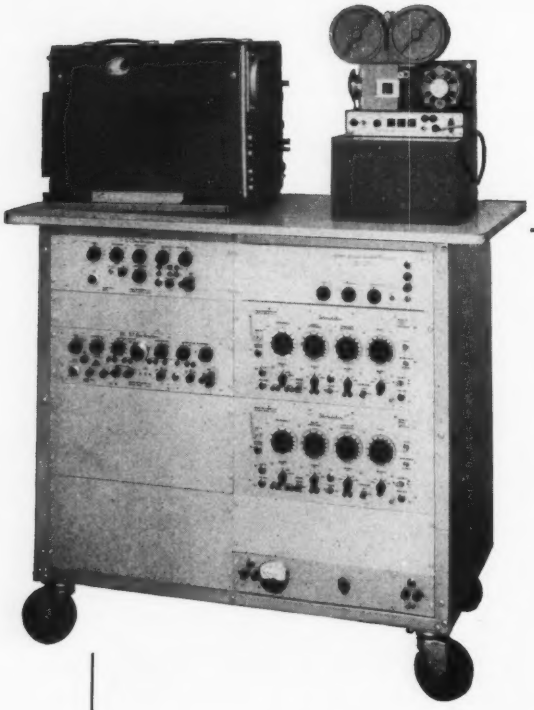

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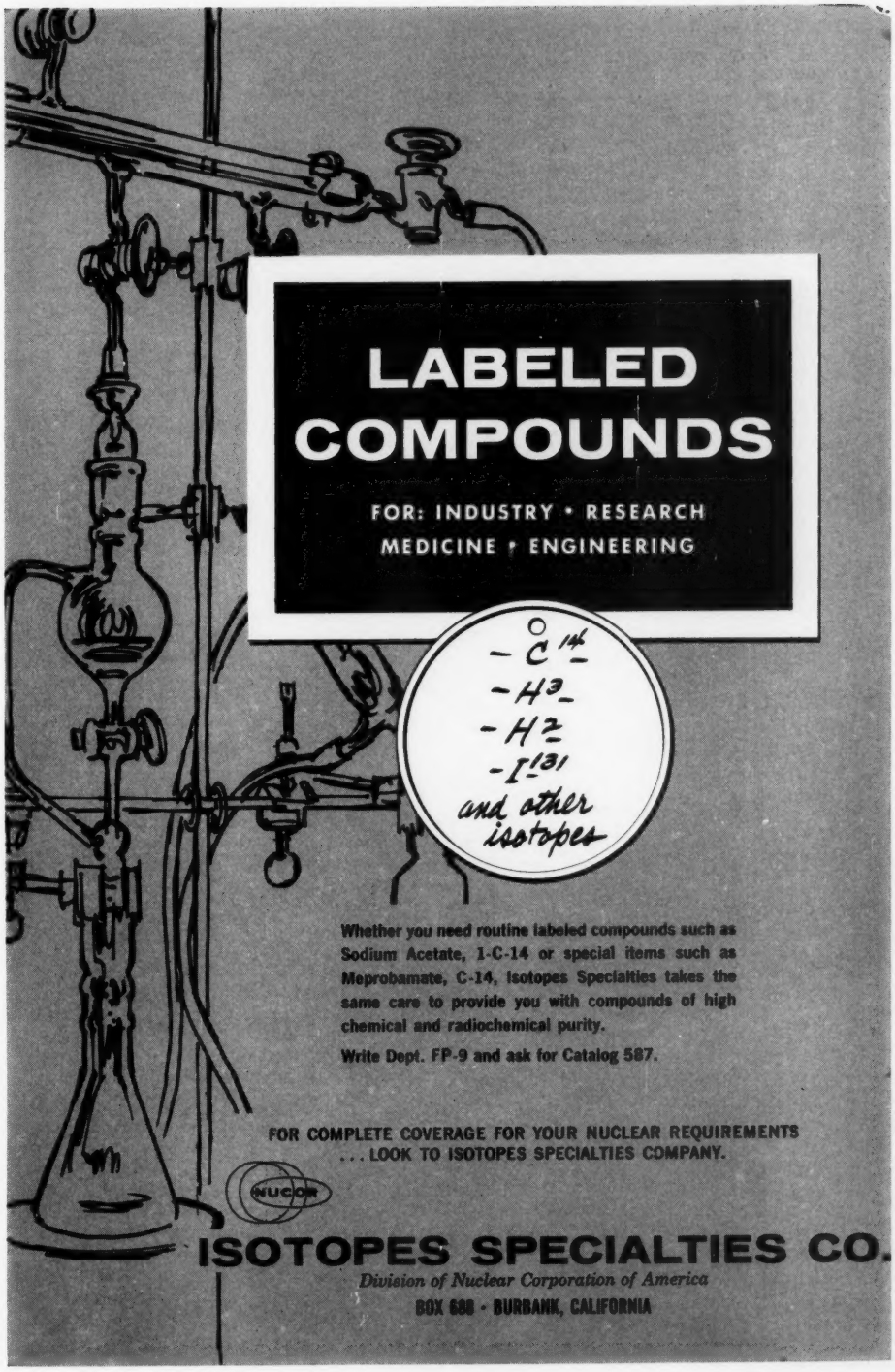
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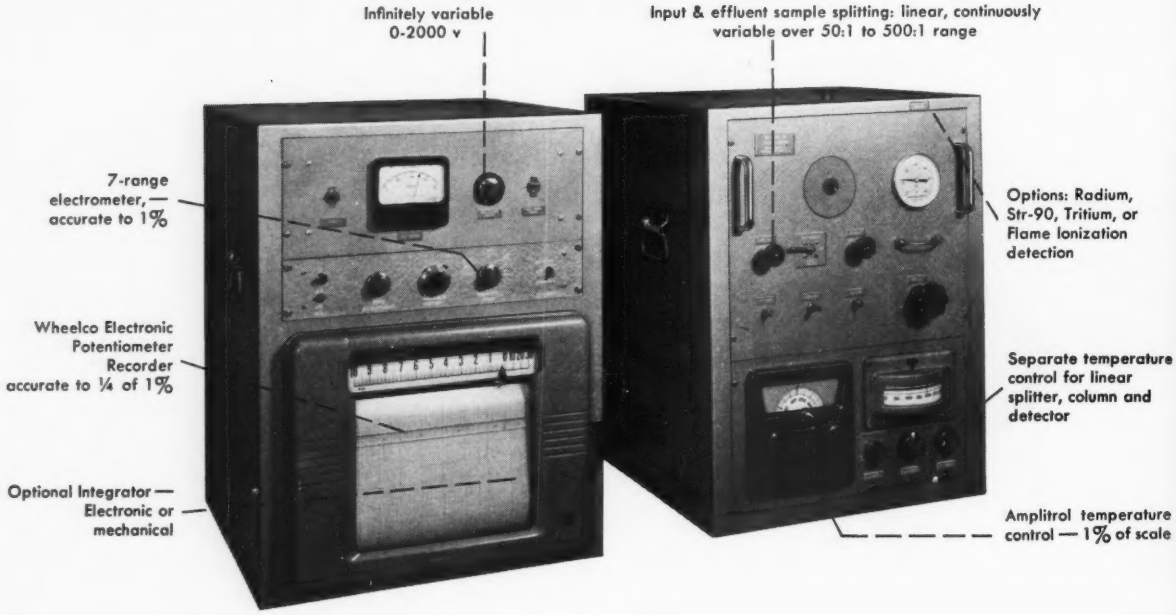
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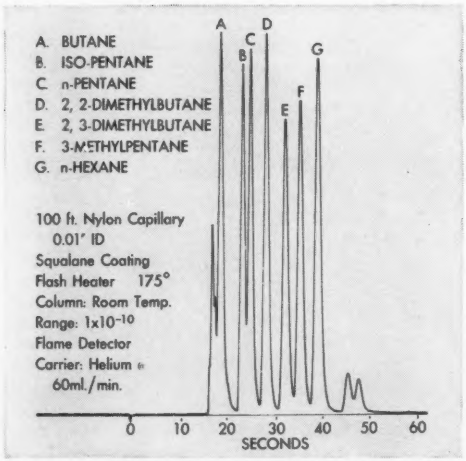

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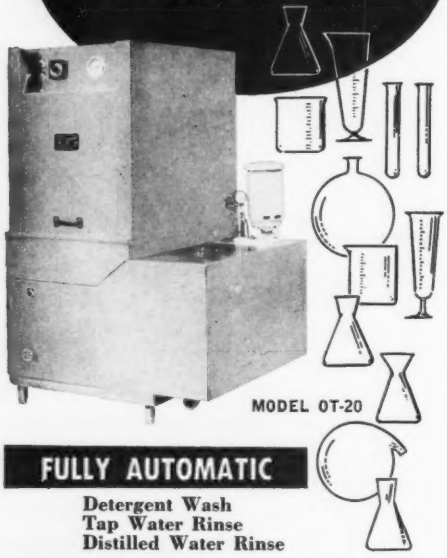

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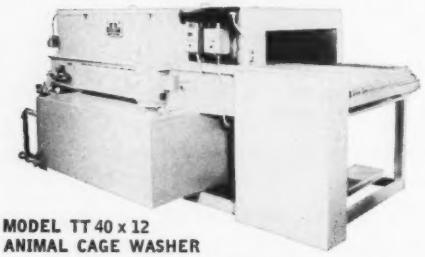

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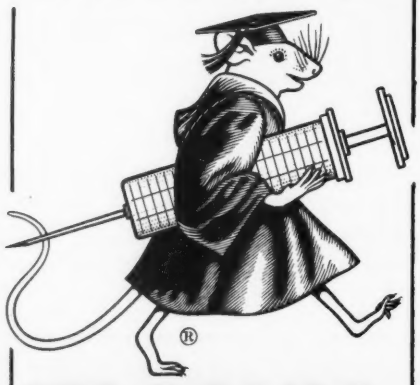

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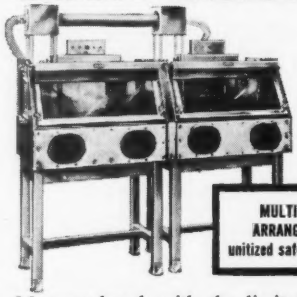

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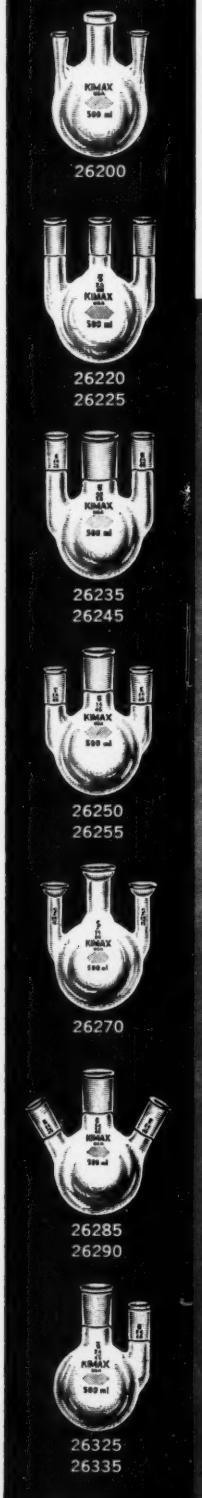

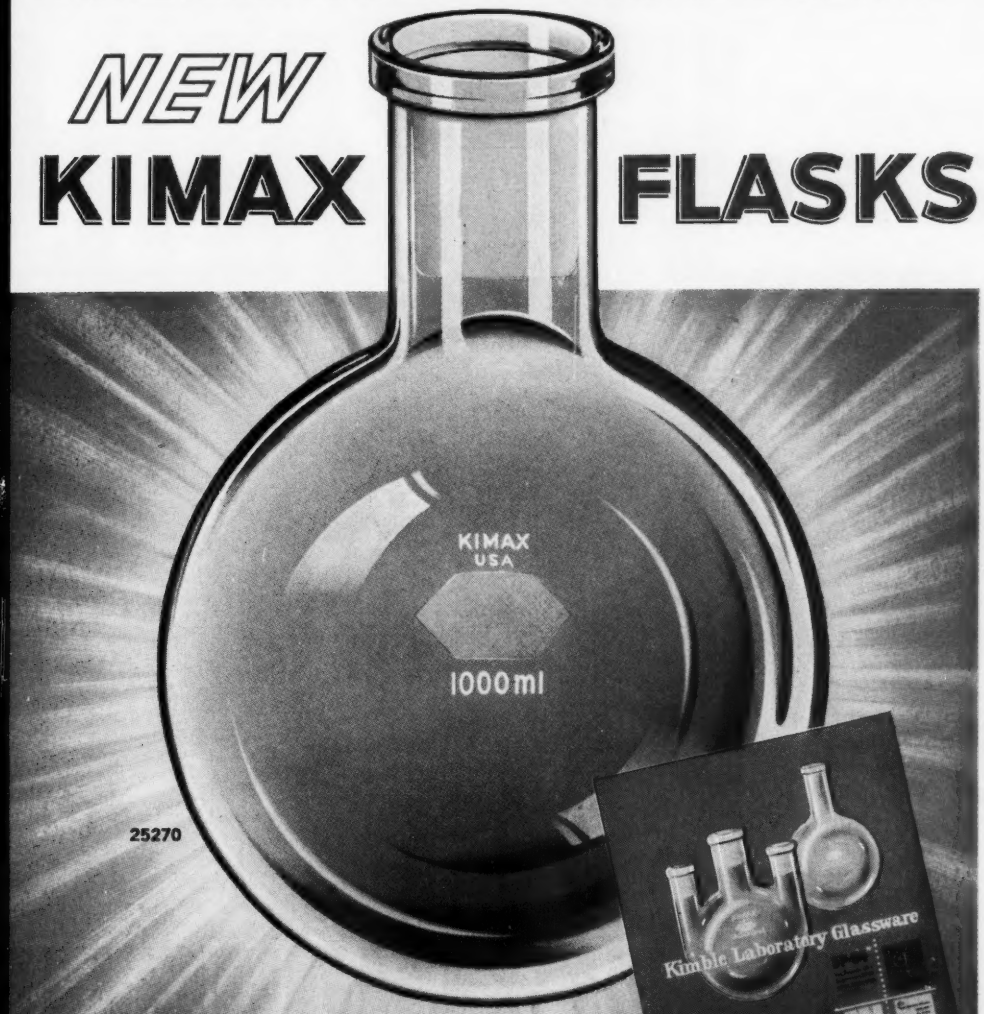

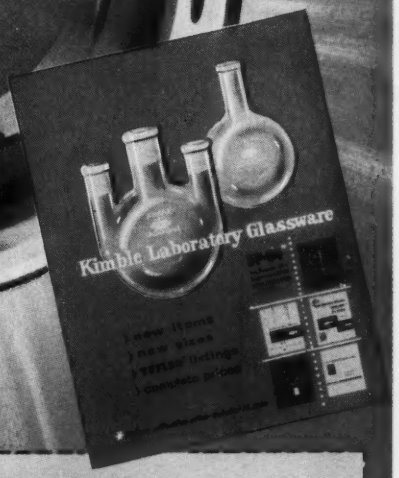

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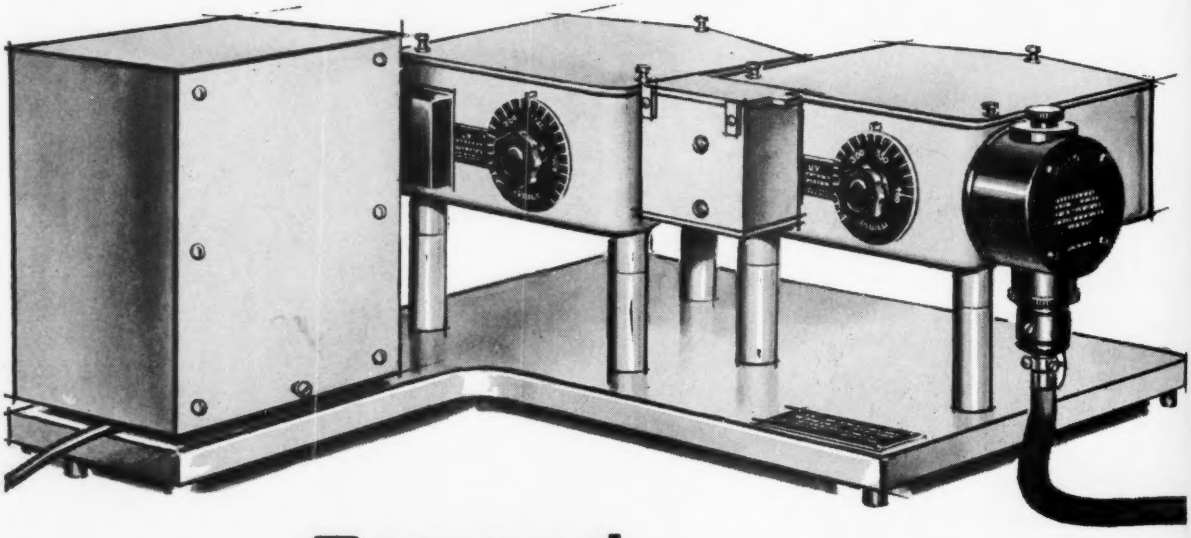

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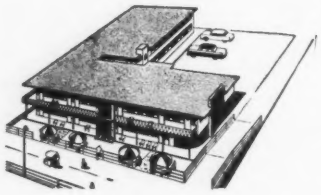

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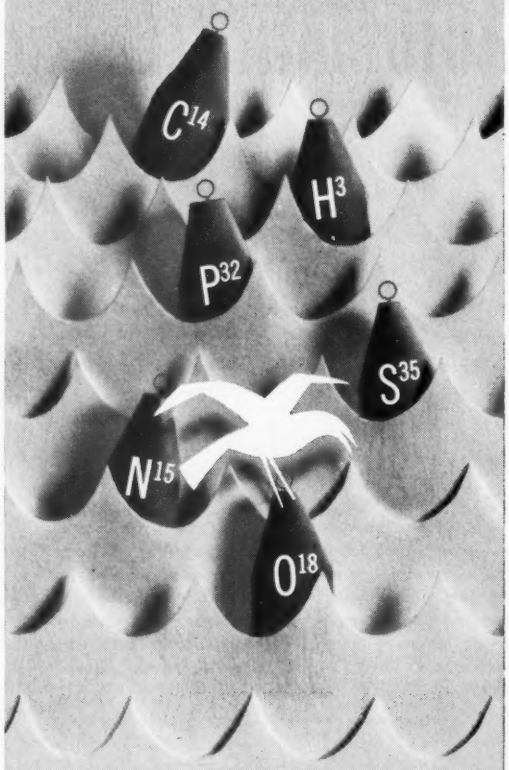

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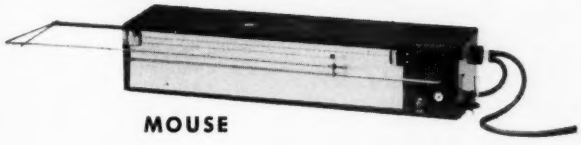

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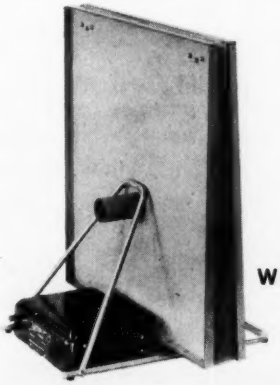

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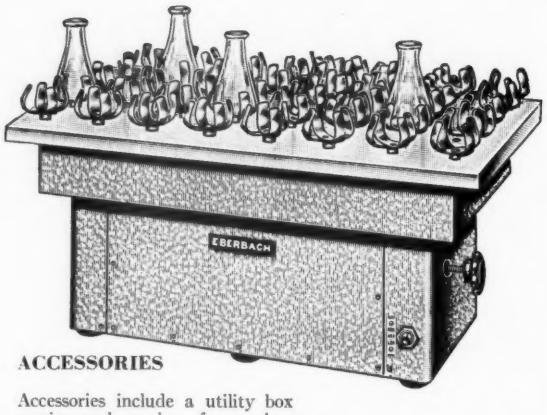

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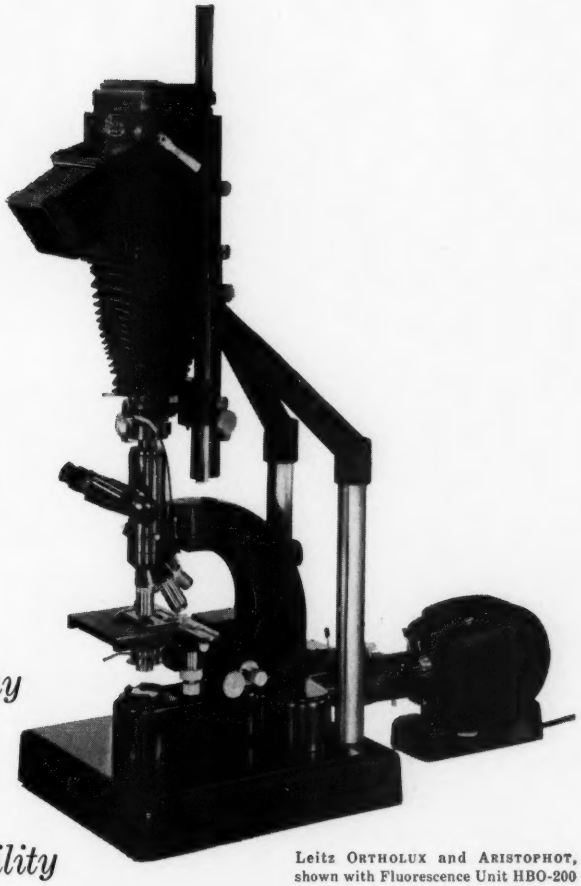

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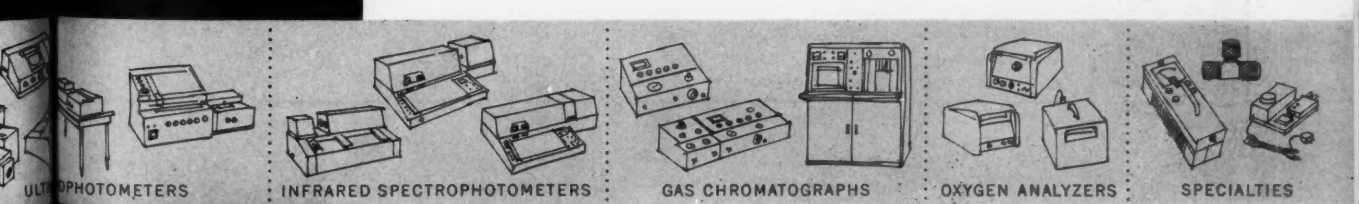

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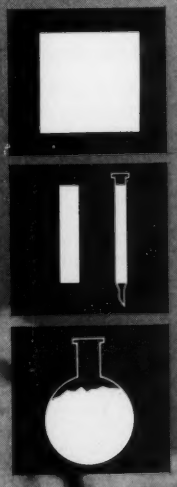

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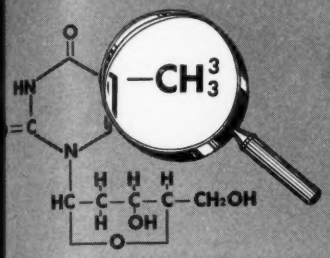

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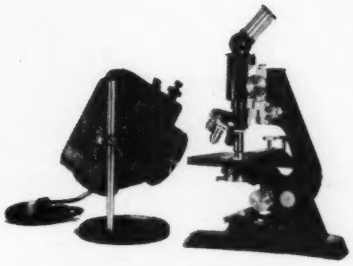

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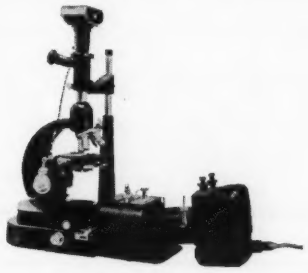

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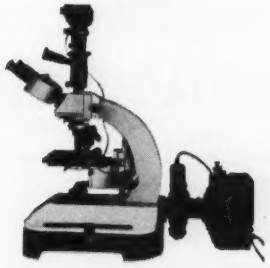

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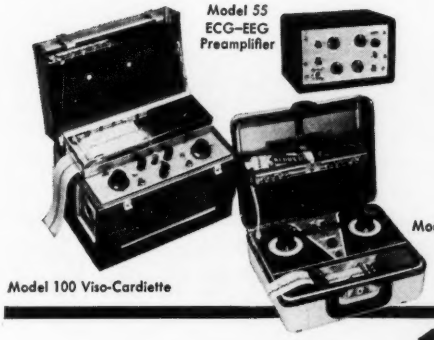

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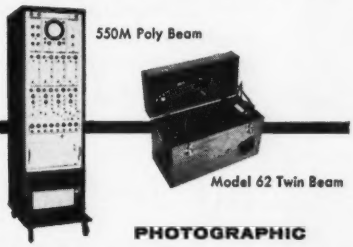

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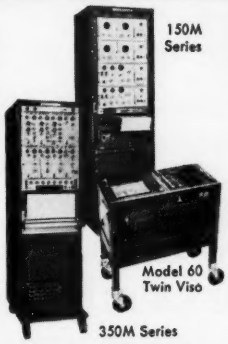

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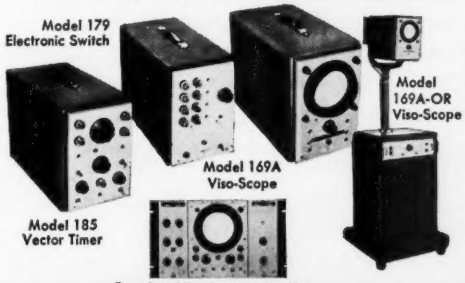

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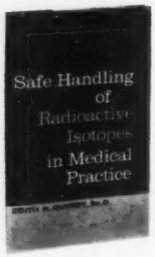

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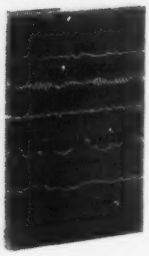

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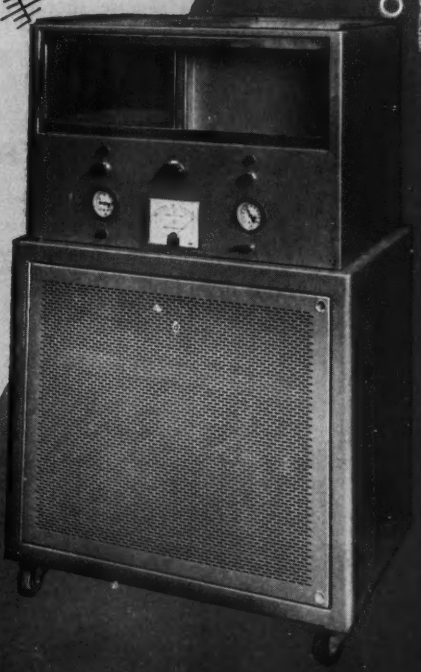

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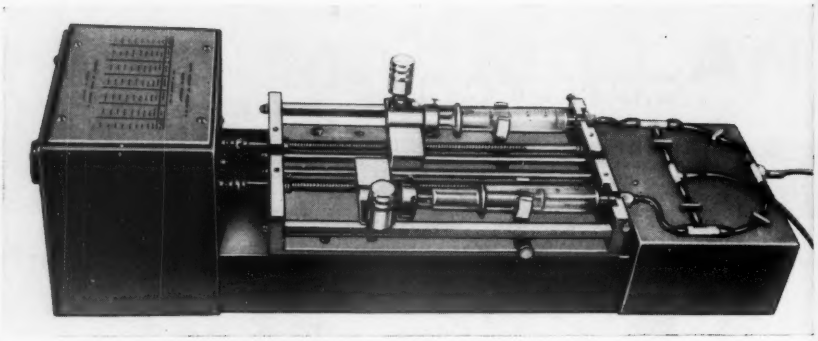

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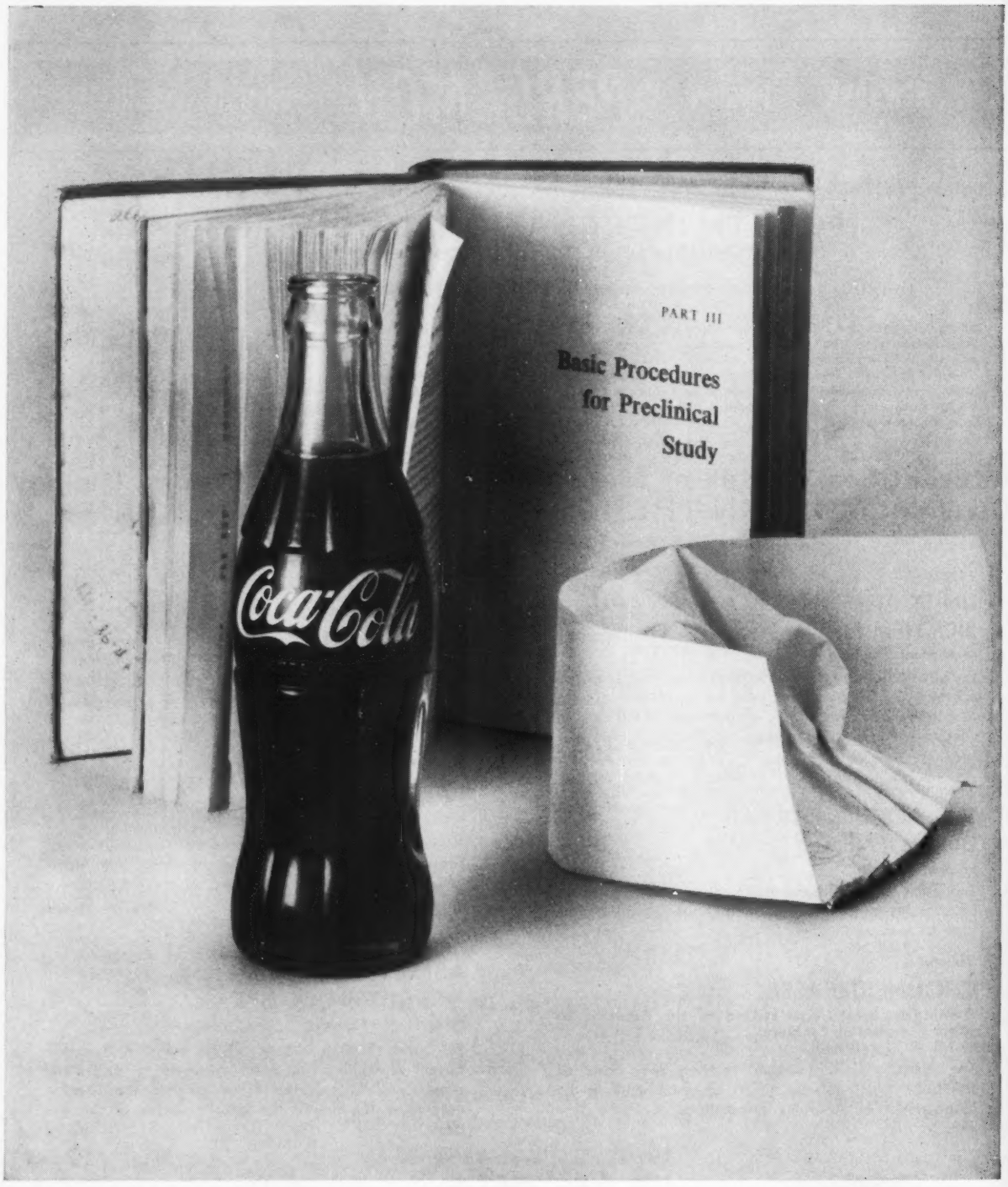

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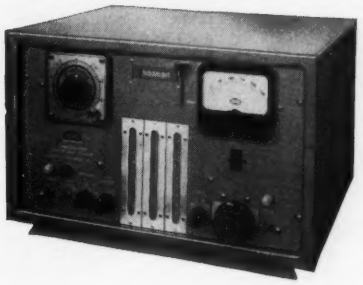

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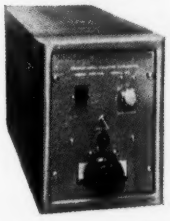

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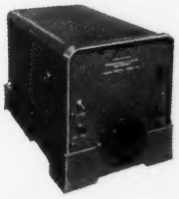

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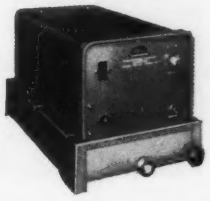

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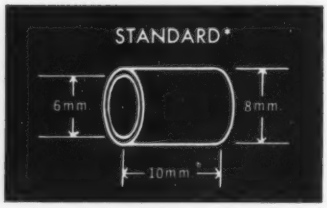

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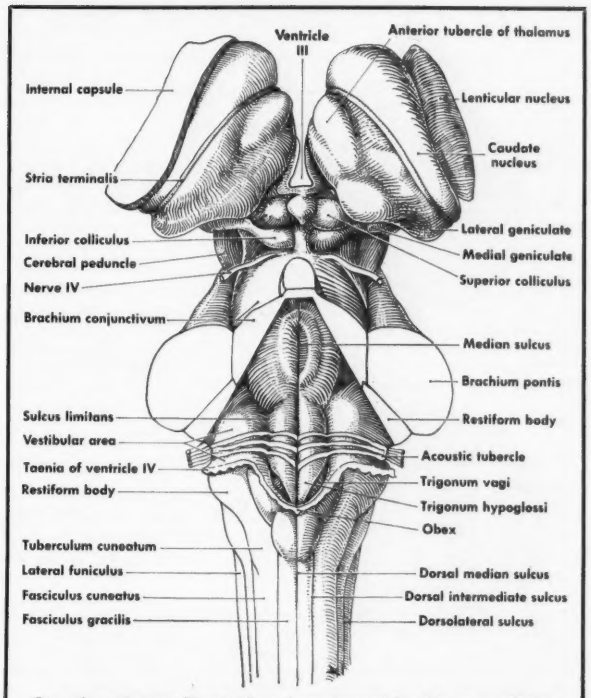

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News & Notes

FORTY-FIFTH ANNUAL MEETING

Atlantic City, New Jersey

April 10–15, 1961

The 45th Annual Meeting of the Federation will be held in Atlantic City, April 10–15, 1961. Because of the volume of papers to be presented, scientific sessions will begin at 9 a.m., Monday, April 10, and WILL CONTINUE THROUGH SATURDAY AFTERNOON, April 15. Federation Headquarters, the Placement Service and many of the scientific sessions will be in Convention Hall. Council rooms and some of the scientific sessions of the Societies will be located in the following hotels: Traymore (Physiology), Ambassador (Biochemistry and Nutrition), Dennis (Pharmacology), Ritz-Carlton (Pathology), Shelburne (Immunology).

The Federation Joint Session will be held in Convention Hall on Tuesday evening, April 11.

As in past years, there will be intersociety sessions and intersociety symposia.

The motion picture session customarily held on Monday evening of the Annual Meeting will not be scheduled at the 1961 Meeting. Instead, motion pictures will be shown continuously during several days of the Meeting.

Motion pictures may *not* be shown in the regular scientific sessions.

REGISTRATION INFORMATION

The registration area will be in the Lobby, Convention Hall, opening at noon on Sunday, April 9. Registration desks will remain open until 11 p.m., Sunday, and from 8 a.m. until 5 p.m., Monday through Saturday.

Members of the Federated Societies and non-member biologists, physicians and others who wish to attend the scientific sessions and exhibits must register for the Meeting. The registration fee is $10. Advance registration is strongly urged. Advance registrants spend only a few minutes in the registration area, even during hours of peak registration. Advance registration forms will be mailed on request from the Federation Office.

To register in advance, mail the registration card with your fee of $10 to the Federation Office, 9650 Wisconsin Avenue, Washington 14, D. C. Advance registration will be accepted until March 20. A validated receipt will be mailed to you. BRING THIS RECEIPT WITH YOU TO THE ANNUAL MEETING. Upon presentation of this receipt at the *Advance Registration Desk*, you will receive your official badge which *must* be worn for admission to any activity including scientific sessions, exhibits, and the Placement Service.

Copies of FEDERATION PROCEEDINGS, March 1961 (Part I—Abstracts, Part II—Program) and tickets to special functions will be on sale in the registration area, Convention Hall. A visible directory of registrants, an information desk, and a message center for distribution of mail and personal messages will also be located in the Lobby, Convention Hall.

(Continued on page lxii)

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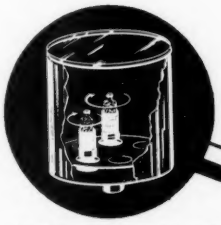

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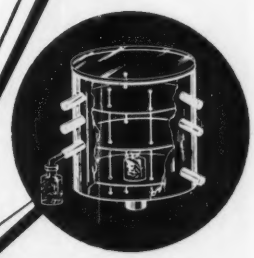

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(Continued from page lx)

TRANSPORTATION FACILITIES

The Federation is making a special effort to facilitate travel into and out of Atlantic City. During the Annual Meeting, shuttle buses will travel between the Philadelphia Airport and Atlantic City on a regular schedule, with a one-way fare of $1.54. The Federation bus schedule will be mailed on request from the Federation Office. To ensure good connections, refer to the bus schedule, and *make your travel reservations early.*

HOTEL RESERVATIONS

A Housing Bureau will serve members and non-members attending the Federation Meeting. The official form for hotel reservations must be used to obtain special Federation hotel rates in Atlantic City. These forms will be mailed on request from the Federation Office. Please address hotel forms to: 1961 Federation Meeting, Housing Bureau, 16 Central Pier, Atlantic City, New Jersey. Confirmations will be forwarded directly from the hotels. The Atlantic City hotels have assured us that rooms will be delivered at the price confirmed.

ANALYSIS OF ATTENDANCE

Attendance and the number of papers read at the Federation Annual Meeting are increasing each year. This makes necessary careful studies of the structure and organization of the scientific sessions, and of the duration of the Meeting. Data are needed from those attending the 1961 Meeting on the variety of sessions they attend, and particularly on the cross-overs among the sessions of different Societies.

At the registration desk you will be given some form of identification denoting Society of major interest, and a return postal card on which you will be asked to check the sessions you actually attend. Will you please cooperate in this important evaluation.

DEADLINE FOR ABSTRACTS

December 15, 1960 is the deadline date for receipt of abstracts by the Society Secretaries. Abstracts received on or after December 16, 1960 will not be accepted.

EXHIBITS

Scientific and institutional exhibits will be located on the Main Arena Floor, Convention Hall. They will be open from 8 a.m.–5 p.m. on Monday, April 10, and from 8:45 a.m.–5 p.m. on Tuesday, April 11 through Friday, April 14. Member exhibits will not be accepted for the 1961 Annual Meeting.

For information and application forms for institutional exhibits, write to the Federation Convention Office, 9650 Wisconsin Avenue, Washington 14, D.C. January 9, 1961 is the deadline date for receipt of completed application forms.

SPECIAL FUNCTIONS

The Federation Office will assist with arrangements for meal functions and meetings of informal groups. Reservation forms and information are available from the Federation Office, 9650 Wisconsin Avenue, Washington 14, D.C.

(Continued on page lxiii)

(*Continued from page lxii*)

If the time and location of these events are to be listed in the Program, all arrangements must be completed by January 30, 1961.

Tickets to special functions will be on sale in the registration area, Lobby, Convention Hall.

WOMEN'S HOSPITALITY LOUNGE

A Women's Hospitality Lounge, located in the Founders Room of the Dennis Hotel, will be open throughout the Annual Meeting. Upon payment of a registration fee of $1.00, wives and other guests of registrants are invited to use the Lounge as a meeting place and social center from 9 a.m. to 5 p.m., Monday, April 10, through Saturday, April 15. No special entertainment will be scheduled for the ladies, but free coffee service, card tables and cards will be provided. Women may register in the Lounge from 9 a.m. to 5 p.m. on Monday and Tuesday, April 10 and 11, and thereafter in the main registration area, Convention Hall.

PLACEMENT SERVICE

The Placement Service will be located on the Main Arena Floor, Convention Hall. Facilities will again be available for employers wishing to schedule personal interviews with candidates. To use these facilities, *both* employers and candidates must have paid the registration fee for the Annual Meeting and must also be registered with the Placement Service.

The February 1961 List of Candidates will be the final list issued before the Annual Meeting. Candidates who wish to be included in this bulletin must file their applications by January 31. Advance registration of candidates who wish to use the Placement Service facilities but who do not wish to be listed in the quarterly bulletin will be accepted until March 31.

The March 1961 List of Available Positions will be the final one to be published before the Meeting. In order to have a description of the positions entered in this list, employers must submit completed registration cards before March 1. Advance registration of employers not appearing in the March bulletin will be accepted until March 31.

Employers and candidates may register at Atlantic City, but the procedure will be more time-consuming.

All correspondence regarding this activity should be directed to: Federation Placement Service, 9650 Wisconsin Avenue, Washington 14, D.C.

FUTURE ANNUAL MEETINGS

Future Annual Meetings will be held as follows:

1962: April 16–21, Atlantic City
1963: April 15–19, Atlantic City
1964: April 13–19, Chicago

(*Continued on page lxxviii*)

FORMS AND ANNOUNCEMENTS

Containing specific instructions and rules for participation in

THE 1961 MEETING

may be ordered from the Federation Office by using the coupon below:

To: Federation Office
9650 Wisconsin Avenue, Washington 14, D. C.
Please send the following forms for the Federation Annual Meeting in the quantity indicated:

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INTERSOCIETY SYMPOSIUM ON ANIMAL BEHAVIOR AND NEUROPHYSIOLOGICAL CORRELATES

Introductory Remarks

P. B. DEWS, *Chairman*
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

THIS IS THE THIRD in a series of annual symposia on topics relating to the central nervous system held as part of the program of the Federated Societies under the joint sponsorship of the Physiological and the Pharmacological Societies. The series was initiated by Dr. Hallowell Davis. It has already become traditional that the program should include someone engaged in electrophysiological studies, someone engaged in behavioral studies, and someone engaged in biochemical studies; this symposium follows the tradition. The title 'Animal Behavior and Neurophysiological Correlates' was chosen by Dr. Harold Himwich, who was chairman of the committee comprised of himself, Drs. Ruch and Unna and me, charged with organizing the program. Actually, the program is not noticeably coherent as a whole; the three main topics are relatively independent. No apology is made for this for two reasons. One, that it seems to me that it is much more important that a contributor to an interdisciplinary session should present critically information in his special field of competence than that he should speculate in other areas. Two, that information on significant advances in any area of the biochemistry or physiology of the central nervous system or of behavior should be of interest to students in all other areas. It seems to the present writer that the appropriate forum for presentation of connected accounts of significant advances in any of the fields represented by the constituent societies of the FASEB is the platform of symposia at the annual meeting. It is not in the spirit of science to protect these presentations from the ears and eyes of the vulgar by giving them to restricted audiences in exclusive resorts.

Effects of sleep and waking on spontaneous and evoked discharge of single units in visual cortex

EDWARD V. EVARTS

Laboratories of Neurophysiology and Clinical Science, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

Since the discoveries of Berger (6), Adrian and Matthews (2), and Bremer (8–10) concerning the characteristics of cerebral electrical activity associated with sleep and waking, there have been numerous studies of the effects of sleep and arousal on brain potentials in both man and lower animals. In large part these studies have involved macroelectrode recordings of the summed activity of populations of cerebral neurons. In considering the elimination of slow waves from these recordings during arousal, Adrian and Matthews (2) suggested that the slow waves were based on synchronization of the activity of individual neurons, and that their disappearance was the result of the desynchronization of this activity. Reduction of a variety of evoked cortical potentials (4, 11, 13, 14, 16, 18, 21, 27, 34, 49, 50, 54, 58) has also been shown to occur during arousal. The reduction of these evoked potentials has generally been viewed as the result of desynchronization and occlusion. With the advent of techniques which have permitted studies of the activity of individual cerebral neurons, it has become possible to obtain additional data concerning mechanisms underlying the appearance and disappearance of spontaneous slow waves and of evoked potentials in macroelectrode recordings. On the basis of microelectrode recordings, Adrian, who proposed synchronization and desynchronization as the bases for these alterations, was one of the first to question the adequacy of this explanation. In studies of the electrical activity of the olfactory bulb, Adrian (1) observed that a strong olfactory stimulus abolished the spontaneous rhythmic activity of the bulb. He stated that "After a strong olfactory stimulus the rhythm is abolished and it is built up again gradually, but its absence is associated with a reduction in the activity of the olfactory tract and its return with an increase of activity. In this case therefore the break up of the rhythm seems to involve a decrease in the activity of the cells, and it is not merely that their activity is no longer synchronized."

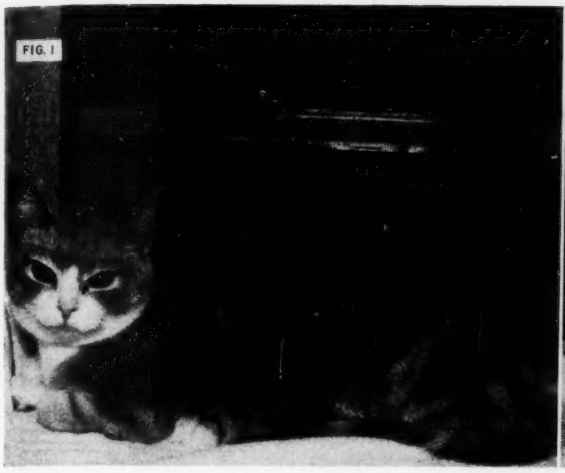
Reduction of neuronal discharge in association with abolition of a slow rhythm has also been observed in the cortex. Whitlock *et al.* (58) found that units in the motor cortex which discharged in association with spindle waves show arrest of discharge when the spindle waves are eliminated by arousal. These authors suggested that arousal may be associated with inhibition of discharge in these neurons.

More recently, it has become possible to record the activity of single neurons in intact unrestrained preparations. Techniques devised by Hubel (28) and Jasper (33) allow studies of unanesthetized intact preparations in which observations of behavior may be related to activity of single neurons. It was in this type of preparation that Jasper and his colleagues obtained further evidence that arousal is associated with inhibition of discharge in many cortical neurons. In referring to Ricci's observation on arrest of single unit discharge in the temporal lobe by arousal, Jasper points out that "This can hardly be called 'ascending activation' unless one includes inhibition in the processes activated." (33)

The studies referred to above have indicated the importance of inhibition as one factor underlying differences in cortical electrical activity between sleep and waking. The present study, employing a preparation similar to that employed by Hubel, was carried out to obtain additional information concerning differences in cortical electrical activity during sleep and waking, and to determine the relation of alterations of evoked activity to associated alterations of spontaneous activity.

METHODS

Microelectrodes. Figures 1 and 2 illustrate the type of preparation which was employed; figures 3 and 4 show the components of the system which was used for microelectrode recording. An externally threaded stainless steel cylinder with two projections at its base was placed so that the projections were beneath the skull. A small extension at the margin of the trephine hole allowed the



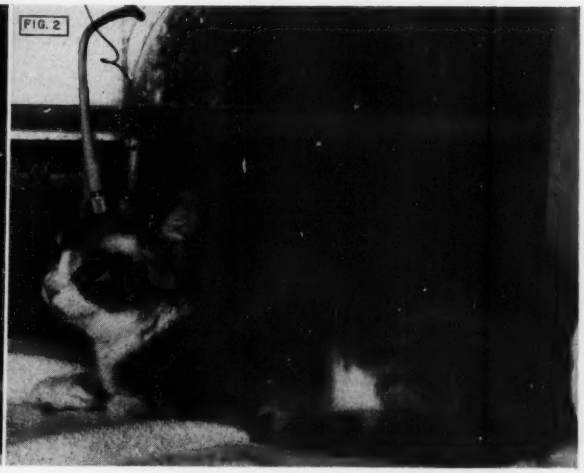
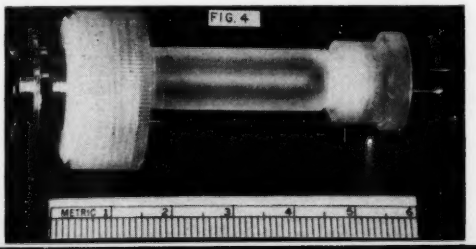


FIG. 1. Cat with contact occluders in place.

FIG. 2. Cat following attachment of gross electrode cable and microelectrode carrier. The anterior device is the Sheatz pedestal, in which are fixed the terminals of electrodes implanted for stimulation of the lateral geniculate radiations and for recording the spontaneous slow wave activity from the cortical surface. The posterior device is the microelectrode carrier. The vertically projecting cylinder contains a piston which may be lowered 30 mm, allowing penetration of deep structures in the brain.



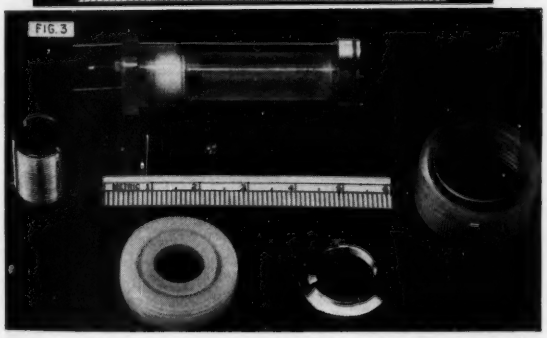


FIG. 3. Components employed in fixation and driving of microelectrode. At top is cylinder with hydraulically driven piston, modified after the one developed by Hubel. At left is stainless steel cylinder whose basal projections are placed beneath the skull. Steel ring at bottom right is screwed down on this cylinder until bone is firmly held between ring (above the bone) and projections at base of cylinder (beneath the bone). Nylon ring at lower left, internally and externally threaded, is attached to steel cylinder and serves as base for microelectrode drive (top) which is held onto this base by internally threaded collar (right).

FIG. 4. Assembled components of microelectrode system.

projections at the base of the cylinder to be placed beneath the skull. An internally threaded ring was put onto the cylinder and tightened so that the bone was grasped firmly between the projections at the base of the cylinder and the ring. The center of the cylinder was placed at Horsley-Clarke coordinates A_2-L_0 in order to allow penetration of either lateral gyrus. Caution was exercised to avoid injuring the dura, since it was found that the intact dura prevented injury to the subjacent cortex. Electrodes were inserted into the lateral gyrus between Horsley-Clarke coordinates A_4-A_0 and L_2-L_3.

The hydraulically driven micromanipulator, which was modified after that of Hubel (28), was placed eccentrically over the cylinder, allowing the electrode to be inserted at any point on a circle whose radius was 3 mm and whose center was at the center of the cylinder which was fixed to the skull. Oil-filled polyethylene tubes connected to the two chambers of the micromanipulator, and running into a room adjacent to the one in which the preparation was placed, allowed the electrode to be raised or lowered in small steps. Tungsten microelectrodes were prepared according to the method of Hubel (29). Penetration was limited to a depth of 1.8 mm.

Macroelectrode implantation. At the time of implantation of the cylinder which was to carry the micromanipulator, macroelectrodes were implanted for electrical stimulation of lateral geniculate radiations and for recording the spontaneous activity of the visual cortex. The technique of Sheatz (55) was employed in fixation of these electrodes. Three .009 inch diameter enamel-insulated stainless steel wires with tips 1 mm apart were placed in the lateral geniculate radiations bilaterally. The spontaneous slow wave activity was recorded between an extradural electrode at the margin of the cylinder which carried the micromanipulator and an electrode placed in the bone of the occipital crest. The Sheatz pedestal in which the external terminations of these

macroelectrodes were fixed was grounded and served as a reference for the microelectrode.

Experimental conditions. Three days elapsed between the surgical procedures and commencement of recording. Cats were placed in a slowly revolving treadmill during the night prior to recording sessions. In the morning they were fed and placed unrestrained on a soft pad in a 2 ft. x 3 ft. x 3 ft. cage within a soundproof room equipped with a one-way window. In studies of responses to lateral geniculate radiation stimulation opaque contact occluders (45) were placed in both eyes. These occluders extended well beyond the limbus and excluded all light save that which might reach the retina by penetrating bone, soft tissue, and sclera. Such light as did impinge upon the retina must have been of approximately equal intensity during sleep and arousal. In studies of unit responses to retinal illumination 10 msec. flashes were delivered to the contralateral eye by a small neon bulb which had been attached to a translucent contact occluder. The external surfaces of the contact occluder and neon bulb were made opaque to exclude external light. The pupil was dilated prior to placement of the light source. The ipsilateral eye was covered with an opaque contact occluder. A period of dark adaptation of approximately 30 minutes was allowed prior to the start of recording.

Recording and analysis of data. A flexible lead connected the microelectrode to a Bak amplifier (5) which was suspended above the preparation. The suitably amplified output of the Bak amplifier was displayed on a recurrently sweeping cathode ray beam and recorded on moving film. A time constant of 2 msec. was employed to eliminate slow wave activity from the microelectrode record. Spontaneous slow wave activity from the cortical surface was recorded with an ink-writing oscillograph. Observations concerning the behavior of the preparation were written on the EEG record; such observations are particularly important in studies of sleep and waking, since observation of the EEG alone does not allow distinction between arousal and that phase of sleep in which slow waves are absent (15, 31, 32, 35, 36). Following completion of recordings the EEG and behavioral notes were used to determine presence or absence of sleep. For the present report, analyses of unit activity have been restricted to periods of sleep with pronounced slowing of spontaneous activity and to periods of waking as indicated by the behavior of the cat and by the absence of slowing in the EEG. Unit activity occurring in association with sleep but in the absence of EEG slowing will not be described here. Thus, for the present report, the term sleep will in all cases mean sleep with EEG slowing. Units were selected for analysis only if they were recorded during successive periods of sleep and waking.

Of 286 units studied 90 were observed for sufficiently prolonged periods of sleep and waking to allow analysis of their records according to the criteria indicated above. These 90 units may be divided into two groups. The first group (41 units) was derived from studies of the effects of sleep and waking on responses to lateral geniculate radiation stimulation. The second group (49 units) was derived from studies of effects of sleep and waking on responses to retinal illumination. Of the entire group of 286 units 190 were initially positive and 96 initially negative. In the sample of 90 units selected for analysis 52 were initially positive and 38 initially negative. The initially positive units were, as observed by previous investigators (3, 24, 47, 51), of relatively great amplitude (1–10 mv) and in most cases showed the A-B break of Fuortes *et al.* (23). The initially negative units were rarely greater than 1 mv in amplitude. The inversion of initial sign was commonly observed in the sequence described by Mountcastle *et al.* (47) and by Phillips (51). In presenting the results below no distinctions will be drawn between initially positive and initially negative units. Separate analyses of these two groups have been carried out, however, and have shown that the results to be presented in this report were true of both groups of units. In spite of the qualitatively similar effects of sleep and waking in these two groups of units, comparisons between the groups were consistent with the findings of Mountcastle *et al.* (47) concerning reduced excitability in the initially positive units.

RESULTS

Activity evoked by geniculate radiation stimulation. A previous study in cats with chronically implanted electrodes has shown that the amplitude of the response to lateral geniculate radiation stimulation is reduced during waking as compared to sleep (22). Desmedt and La Grutta (16) have described a similar effect of arousal in the encephale isolé. Reductions of a variety of additional evoked cortical responses by arousal have previously been described by other investigators (4, 11, 13, 14, 16, 18, 21, 27, 34, 49, 50, 54, 58). One purpose

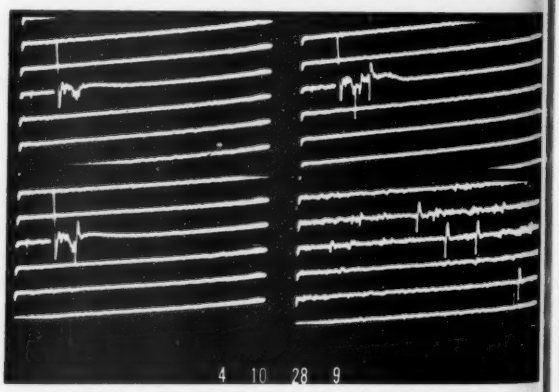


FIG. 5. Response of initially negative unit to lateral geniculate radiation stimulation. Upper left shows shock artifact and gross response without unit discharge. Lower left and upper right show unit discharging once and twice, respectively, in response to radiation stimulus. Lower right shows spontaneous discharge. Sweep duration is 25 msec. In this and all subsequent illustrations, positive is up and time advances from left to right and from below to above.

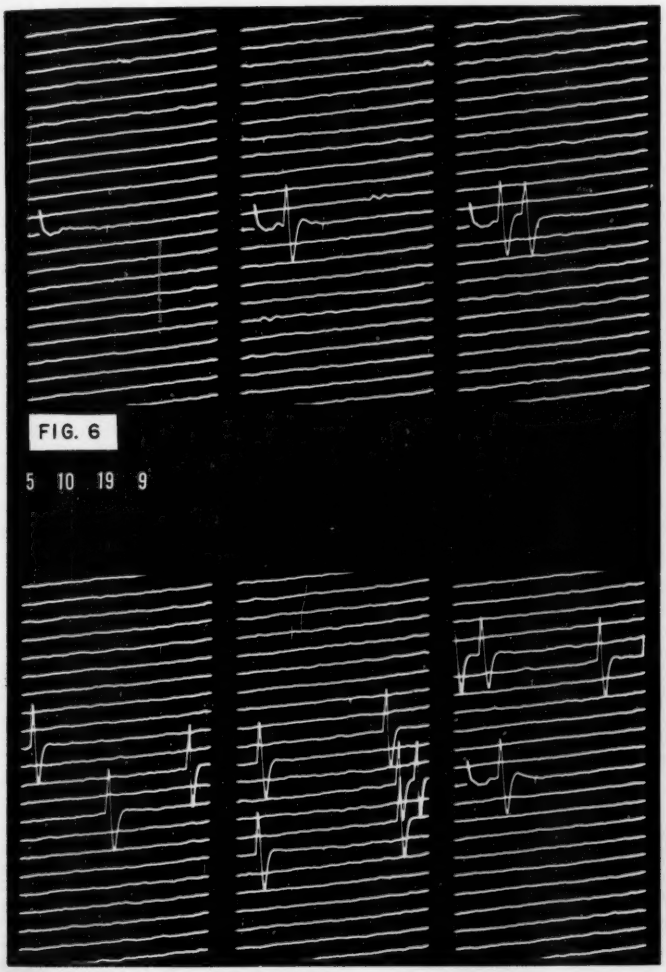


FIG. 6. Response of initially positive unit to lateral geniculate radiation stimulation. Above are shown variations of response to a constant geniculate radiation stimulus, which evokes from 0 to 2 spikes. Below are shown spontaneous discharges in absence of any stimulus (left and center) and following response evoked by geniculate stimulus (right). Sweep duration is 25 msec.

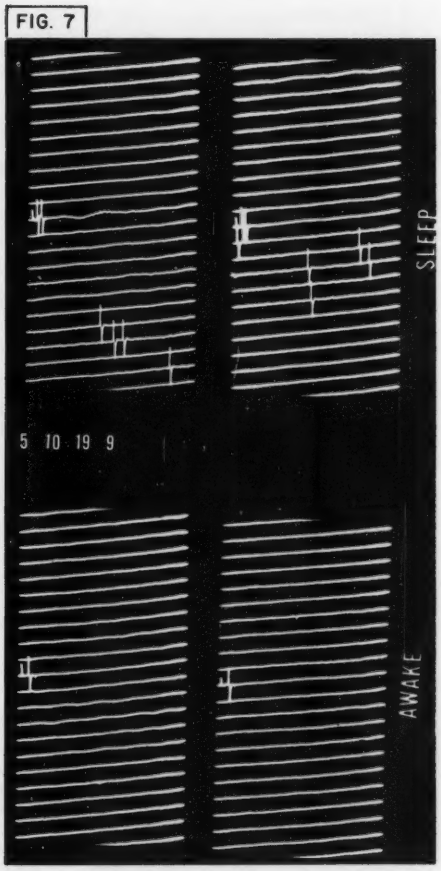


FIG. 7. Reduction of evoked discharge and arrest of spontaneous activity during waking. The initially positive unit illustrated in fig. 6 is shown here (at reduced gain and on 100 msec. sweep) with 2 spikes/stimulus and occasional spontaneous discharge during sleep, and with 1 spike/stimulus and arrest of spontaneous activity during waking.

of the present set of observations was to determine the alteration of unit activity which might correspond to the above mentioned reduction of the gross response to geniculate radiation stimulation during waking. A possible explanation for this reduction of gross response amplitude might be that the elements whose discharge gives rise to the response have more interstimulus spontaneous activity during arousal than during sleep, and that this greater interstimulus activity causes reduced responsiveness to the synchronous afferent volley. It therefore seemed important to observe the effects of sleep and waking on both spontaneous and evoked activity in the same population of neurons, and to determine the relation of changes in evoked to changes in spontaneous discharge.

Supramaximal stimuli of 30 μsec. duration and 0.5–2.5 ma were delivered to the geniculate radiations ipsilateral to the visual cortex from which unit records were to be obtained. Of the 150 units which were investigated during geniculate radiation stimulation, 63 responded to the geniculate stimulus at latencies of 1 to 6 msec. and had a variety of characteristics which identified them as postsynaptic elements. Of these 63 units 16 were observed during successive periods of sleep and waking, and were therefore analyzed. Figures 5 and 6 show responses to geniculate radiation stimulation in two units, one initially negative, the other initially

positive. Of the 16 units, 9 were initially positive and 7 initially negative. Figure 7 shows representative traces (during sleep and waking) of the initially positive unit shown in figure 6. It is apparent that in this unit there is a reduction of evoked activity and an arrest of spontaneous activity during waking. It is clear, in this case, that reduction of evoked activity during waking is not secondary to increased spontaneous interstimulus activity. Figure 8 presents the results obtained in 15 additional units. Of these 15 units, 11 showed reductions of evoked activity (spikes/stimulus) during waking; 10 of these 11 had associated reductions of spontaneous activity. All of the units showing reductions of 50% or more in evoked activity during waking showed reductions of spontaneous activity.

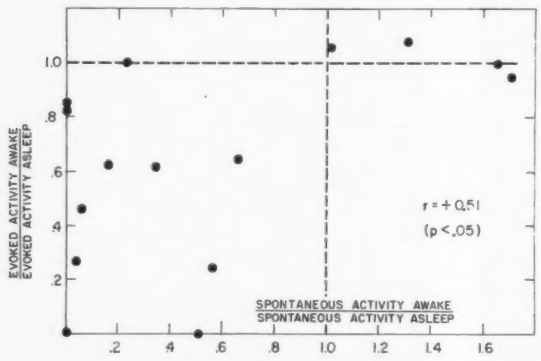
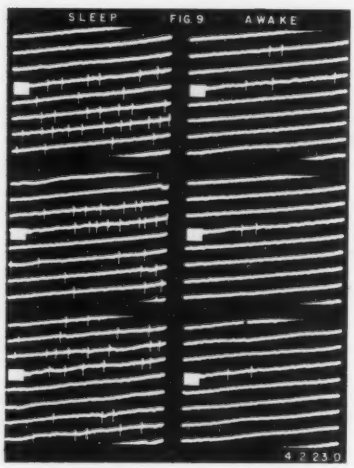
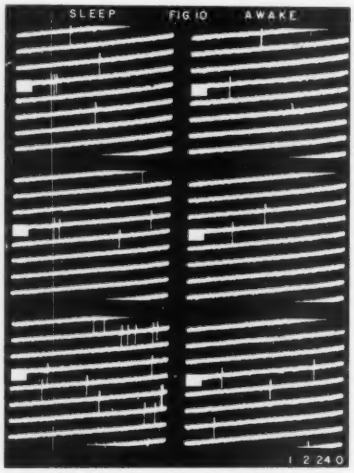


FIG. 8. Effects of sleep and waking on responses to geniculate radiation stimulation. On abscissa is plotted the ratio of spontaneous discharge rate during waking to spontaneous discharge rate during sleep. On ordinate is plotted the ratio of evoked activity during waking to evoked activity during sleep. Points below 1.0 on the ordinate and to the left of 1.0 on the abscissa represent units which had reductions of both spontaneous and evoked activity during waking. Ten units fell into this category. Only 1 unit showed a reduction of evoked activity in association ith an increase in spontaneous discharge during waking.

Activity evoked by retinal illumination. One hundred and thirty-six units were observed during retinal illumination. The photic stimulus was a 10 msec. flash delivered every 2.5 sec. to the contralateral eye. Of the 136 units 84 were initially positive and 52 initially negative. Forty-nine of the group of 136 units were studied during sleep and waking. Of these 49, 17 were regularly excited by the photic stimulus. It is with these 17 units that the present section will deal. Eleven of the 17 were initially positive and 6 initially negative. As mentioned previously, the initially positive units were usually of large amplitude and showed the A-B break, whereas the initially negative units were of lower amplitude and failed to show the A-B break.

For each of these 17 units spontaneous and evoked activity were determined for many presentations of the flash during sleep and waking. As in the case of the results reported in the previous section, it was found that when marked reductions of evoked activity occurred during waking, they were associated with reductions of spontaneous activity. In four of the 17 units waking led to reductions of greater than 50% in the number of





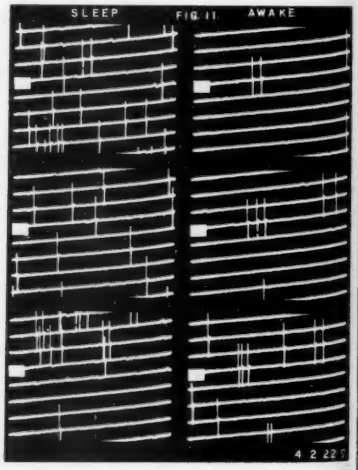


FIG. 9. Reduction of spontaneous activity during waking. The occurrence of a 10 msec. light flash is indicated by white rectangle. During sleep, evoked activity, though shown to be present by statistical analysis of this unit, was obscured by rapid spontaneous discharge. During waking there is marked reduction of spontaneous discharge, and the flash can be seen to elicit two spikes. Sweep duration is 100 msec. Unit is initially negative.

FIG. 10. Reduction of evoked and spontaneous activity during waking. During sleep, the 10 msec. light flash is followed by 1 to 3 spikes. During waking, the flash regularly evokes 1 spike. During waking, the reduction of spontaneous activity is more pronounced than the reduction of evoked activity. Sweep duration is 100 msec. Unit is initially negative.

FIG. 11. Increase in evoked activity associated with decrease of spontaneous activity during waking. During sleep, evoked activity, though present, is of small amount and is obscured by spontaneous discharge. During waking, the flash regularly evokes multiple discharge, and spontaneous activity is reduced. Sweep duration is 100 msec. Unit is initially positive.

spikes/stimulus. In three of these four units there were even more marked reductions of spontaneous activity, while in the fourth there was a slight (20%) increase in the spontaneous rate of discharge. Figures 9 and 10 illustrate the alterations of evoked and of spontaneous activity in two units showing reduction of both evoked and of spontaneous activity during waking. It may be seen that in both of these cases the reduction of spontaneous discharge is more marked than the reduction of evoked discharge.

Three of the 17 units showed marked (at least two-fold) and consistent increases in evoked activity during waking. In these three units there was not a corresponding increase of spontaneous discharge. Figure 11 illustrates the marked increase of evoked discharge which occurs in some units during waking. In the unit illustrated in figure 11, the spontaneous activity showed reduction in association with the increase of evoked activity.

The remaining 10 of these 17 units failed to show marked changes in evoked discharge during arousal. It is apparent that units of this sample showed both increases and decreases of evoked discharge with arousal. If, however, one considers the relation between alteration of spontaneous and of evoked activity during arousal, a somewhat more consistent picture emerges. One way to consider the relation of evoked and of spontaneous activity involves expressing the evoked activity as an 'alteration' of spontaneous discharge rate induced by the stimulus. For example, consider a unit which shows an average of three spikes in the interval between 20 and 40 msec. after the initiation of the photic stimulus (and is thus discharging at a rate of 150/sec. during this particular interval). If this unit has a spontaneous discharge rate of 3/sec., one may say that the stimulus has led to a 50-fold increase in the rate of discharge of the unit. It is clear that if the evoked response remains constant while the spontaneous activity is reduced, the stimulus will have caused a greater change in the rate of discharge of the unit. A similar effect will result from an increase in the evoked discharge without change in the spontaneous activity. Thus, if, in the example referred to above, the unit shows a reduction of spontaneous activity from 3/sec. to 1/sec. while continuing to discharge at a rate of 150/sec. during the specified interval after the stimulus, the stimulus may be said to have become more effective in 'altering' the discharge rate of the unit. During the period of 3/sec. spontaneous activity the stimulus caused a 50-fold increase in rate of discharge, whereas the same stimulus caused a 150-fold increase in rate of discharge when the spontaneous discharge had decreased to 1/sec. Table 1 lists the effect of the photic stimulus on rate of unit discharge during sleep and waking in 17 units. The table shows the ratio of the rate of discharge in a given interval (usually 20–40 msec.) following the initiation of the stimulus to the rate of discharge prior to the stimulus. It may be seen that for a majority of units this ratio is greater during the waking state than during sleep. In only one unit of the group was waking associated with reduction of this ratio to one-half or less the ratio during sleep. In six of the units the ratio became at least twice as great during waking as it had been during sleep. Examination of the results obtained with responses to electrical stimuli applied to the geniculate radiations reveals a similar trend in the effect of waking on this ratio in the case of these responses.

TABLE 1. *Ratios of Evoked to Spontaneous Discharge During Sleep and Waking*

Unit No.	Sleep	Waking	Unit No.	Sleep	Waking
5-2-12	.81	12.65	10-2-27	6.59	5.87
4-2-22	1.98	69.94	10-2-11	7.52	5.56
4-2-23	2.39	7.87	11-2-26	8.22	9.79
1-2-27	2.65	2.48	8-3-2	12.26	20.00
13-2-23	3.45	6.02	8-3-1	12.81	14.41
12-3-1	4.72	2.78	118-2-11	15.86	9.12
11B-2-11	5.86	2.19	2-3-2	15.89	116.67
13-3-1	6.07	12.31	5-3-1	16.28	35.50
1-2-24	6.48	11.60			

The figures in columns headed 'sleep' and 'waking' are ratios of the rate of discharge during a brief interval (usually 20-40 msec.) after the flash to the mean rate of discharge during the 500 msec. prior to the flash.

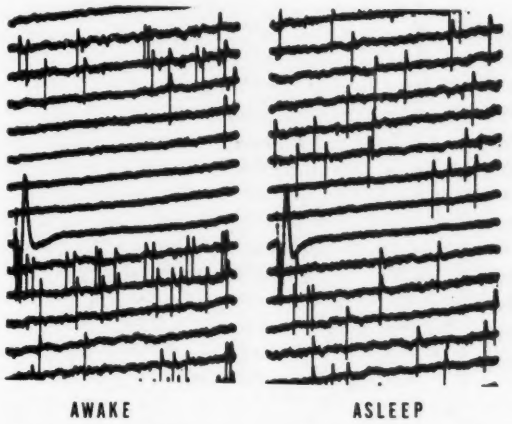


FIG. 12. Inhibition of spontaneous discharge by lateral geniculate radiation stimulus. The slow wave evoked by the stimulus is followed by more prolonged absence of spontaneous activity during waking (left) than during sleep (right). Sweep duration is 100 msec. Unit is initially negative.

A detailed statistical analysis of the present results has not been undertaken at the present time due to the relatively small sample which is available. Thus, the data which have been presented must be viewed as merely suggesting that waking is associated with an increase in the ratio of evoked to spontaneous neuronal discharge.

Inhibitory effects of lateral geniculate stimulation on spontaneous activity. A number of investigators have shown that stimulation of a primary thalamic relay nucleus or radiation pathway inhibits the spontaneous activity of units in the corresponding cortical projection area (7,

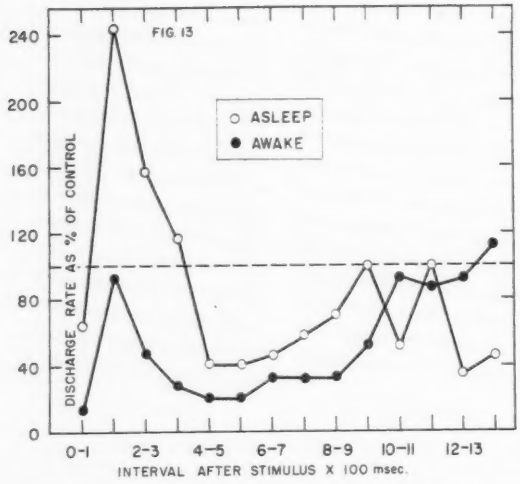


FIG. 13. Inhibition of spontaneous discharge by lateral geniculate radiation stimulus. Rate of spontaneous discharge, as per cent of control rate, is shown for one unit during waking (solid circles) and during sleep (hollow circles). The reduction of spontaneous discharge is more marked during waking than during sleep.

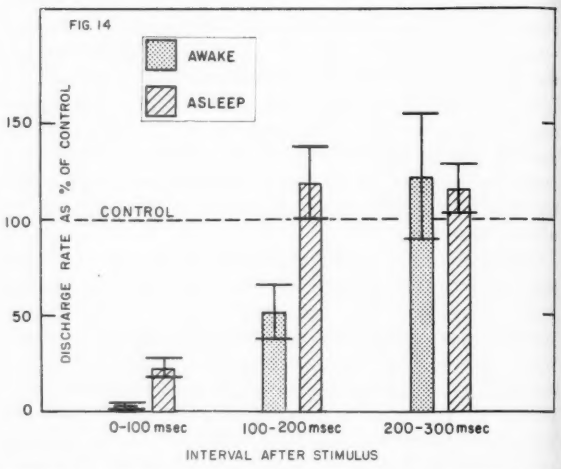


FIG. 14. Averaged results for inhibition of spontaneous activity by geniculate radiation stimulus. The bar graphs show mean rates of discharge, as per cent of control, in 19 units during sleep and waking. The horizontal lines indicate the standard error. Reduction of spontaneous activity was significantly greater ($P < .01$) during waking for the first 2 periods (0–100 msec. and 100–200 msec.).

42, 57); similar inhibitory effects occur following stimuli applied to the cortical surface (7, 37, 39, 52). In view of recent evidence concerning greater activity of inhibitory mechanisms in certain cortical areas during sleep as compared to waking, it seemed worthwhile to determine the effects of sleep and waking on the inhibition of unit discharge in visual cortex induced by lateral geniculate radiation stimulation. Figure 12 illustrates the inhibitory effects of a single geniculate stimulus on spontaneous unit discharge during sleep and waking. Figure 13 is a graphical representation of the inhibitory effect of a single geniculate stimulus during sleep and waking. It is apparent that inhibition is more pronounced during waking than during sleep. The differences in the inhibitory effects of the stimulus on unit activity are consistent with results recently obtained on the recovery cycle of the gross cortical response to lateral geniculate radiation stimulation during sleep and waking (22). In this study, it was found that the recovery curve of this response showed greater subnormality during waking than during sleep. The present observations demonstrate what may be a related phenomenon at the unit level.

Construction of inhibitory curves requires the presence of spontaneous activity. Thus, units which show marked reduction or arrest of spontaneous discharge during waking cannot be used to compare the inhibitory effects of the geniculate radiation stimulus during sleep and waking. Of the 41 units studied extensively during sleep and arousal during electrical stimulation of lateral geniculate radiations, 10 showed arrest or virtual arrest of spontaneous activity during the waking state. Twelve additional units did not provide information concerning differences in the inhibitory effects of the geniculate radiation stimulus between sleep and arousal because their rates of spontaneous discharge were too low to allow determination of the curve on the basis of the number of observations which were available. Inhibitory curves were determined for 19 units during sleep and waking. Figure 14 shows the means and standard errors of the inhibitory effect during periods from 0–100 msec., 100–200 msec., and 200–300 msec. following the stimulus for these 19 units. During the first two of these periods inhibition is significantly greater ($P < 0.01$) during waking. Differences between sleep and waking for the third of these periods (200–300 msec.), and for all remaining points on the curve, are not statistically significant. Inhibition of spontaneous activity was independent of whether or not the unit was excited by the geniculate radiation stimulus, and thus did not represent refractoriness. This independence of inhibition and prior discharge has been previously pointed out by Jung (37).

Spontaneous activity. The spontaneous activity of 90 units was observed during both sleep and waking. Of these 90 units, 48 showed lower rates of discharge during waking than during sleep and 42 showed higher rates of discharge during waking than during sleep. Of the 48 units with reduced activity during waking 17 showed arrest or virtual arrest. The proportion of units showing this marked reduction of spontaneous activity during waking is similar to that observed by Hubel (28) in a previous study of the effects of sleep and waking on spontaneous discharge of units in the visual cortex. Alteration of pattern of spontaneous discharge was observed in many neurons which failed to show marked alterations of over-all spontaneous discharge rates. This alteration of pattern, as previously described by Hubel

(28), takes the form of a reduction of bursts and a general smoothing of the pattern of discharge.

DISCUSSION

Activity has been evoked in units of the visual cortex by two means: electrical stimulation of the lateral geniculate radiations and diffuse illumination of the contralateral retina. Both of these stimuli have certain disadvantages. They were employed because they could be held constant during sleep and waking under the conditions of the present experiment. The electrical stimulus has several disadvantages. First of all, it leads to indiscriminate activation of the radiation fibers and must necessarily obscure the patterns of afferent input which occur in response to physiological activation of retinal elements. Moreover, the characteristics of discharge induced by such a stimulus are entirely dissimilar to that seen in response to retinal illumination. The electrical stimulus gives rise to short high frequency bursts (1–4 spikes) regularly followed by complete suppression of spontaneous activity. Physiological stimulation causes more prolonged discharge of cortical neurons and is not so regularly followed by total elimination of spontaneous discharge. Of advantage, however, are the facts that this stimulus allows positive identification of postsynaptic cortical elements. Changes in responsiveness of cortical elements to such a stimulus may therefore be viewed as the result of changes in cortical excitability and not the secondary result of changes at the level of the retina or lateral geniculate. The photic stimulus, while physiological, has the disadvantage that it is relatively ineffective in exciting discharge of cortical neurons. The results of Hubel and Wiesel (30) show that many cortical units which may be excited by small light sources (either moving or stationary) fail to respond to diffuse retinal illumination.

In spite of the inadequacies of the stimuli employed, the responses which they evoked indicate several alterations of discharge in cortical neurons during waking as compared to sleep. It has been found that reductions of discharge evoked either by flash or by shock are in general associated with reductions of spontaneous activity. This relationship between alteration of evoked discharge and alteration of spontaneous discharge during waking as compared to sleep makes it clear that the reduction of evoked activity is not secondary to increased background activity. In some cases, in fact, reduction of evoked unit discharge was associated with total arrest of spontaneous activity. It would seem that both the reduction of spontaneous discharge and of evoked discharge may result from a higher level of inhibitory input to neurons of the visual cortex during the waking state.

Further evidence of greater activity of inhibitory mechanisms during waking is seen in the more prolonged suppression of spontaneous discharge by lateral geniculate radiation stimulation during waking than during sleep. The basis for the change in inhibitory effects remains to be determined. Several investigators have studied the inhibitory effects of thalamic stimulation (7, 42, 57) and of direct cortical stimulation (7, 37, 39, 52) on unit discharge. The intracellular recordings of Tasaki *et al.* (57) showed that geniculate radiation stimulation led to a suppression of spontaneous discharge, and that this suppression was associated with hyperpolarization of the neuron whose activity was suppressed. Jung (37) has shown that direct stimulation of the visual cortex leads to inhibition of spontaneous unit activity; in these studies it was found that the occurrence of the inhibitory effects was not dependent upon initial discharge of the unit; our observations in this respect confirm those of Jung. In other studies, Jung and Creutzfeldt (40) have shown that inhibition of spontaneous unit discharge occurs in sensori-motor cortex following stimulation at the site of recording or at the corresponding point on the opposite hemisphere. Mechanisms underlying such inhibitory effects have been considered at some length by Phillips (51–53) who found that spontaneous activity of Betz cells could be inhibited either by direct cortical stimulation or by stimulation of the pyramid. Phillips found that surface shocks to the cortex caused hyperpolarization of Betz cells and cessation of spontaneous discharge, whether or not the cells were initially excited by the stimulus. Phillips suggested that the inhibitory effect might be mediated by interneurons analagous to the Renshaw cells of the spinal cord. Branch and Martin (7) observed prolonged inhibition of Betz cell discharge following repetitive stimulation of nucleus *ventralis lateralis*. The mechanism (activation of inhibitory neurons within the cortex) which they propose as underlying this phenomenon would seem to apply equally well to the inhibitory effects observed in the present experiments.

The studies referred to above have demonstrated that inhibitory processes are responsible for the pause in spontaneous discharge which follows direct cortical or thalamic stimulation. The alterations in the duration of this pause of spontaneous discharge of neurons of the visual cortex during sleep and waking indicates that inhibitory processes may be more active during waking than during sleep. This alteration of inhibitory mechanisms during sleep is paralleled by alteration of inhibitory processes by small amounts of an anesthesic agent. The work of Mountcastle (46) and Mountcastle and Powell (48) has shown that afferent inhibition set up by cutaneous stimuli is eliminated by quantities of barbiturate which do not alter the responses of units to excitatory stimuli.

The reductions of evoked discharge which occurred in many units in association with waking may at first glance seem poorly suited to serve perceptual functions. As pointed out by several authors (e.g. 19, 20, 41, 56), however, the detection of a stimulus is based not only on the neuronal activity evoked by the stimulus, but also on the background of spontaneous activity against which the effects of the stimulus must be seen. In the present study, it has been found that many units show

an increase in the ratio of evoked to spontaneous activity during the waking state, whereas a decrease in this ratio is quite rare. The combined results of responses evoked by electrical and by photic stimulation showed that 13 units had a two-fold or greater increase in this ratio during waking, while in only three units was the ratio reduced to one-half or less. This ratio may be thought of as roughly analagous to the signal to noise ratio of communication engineering. It seems possible that the relative increase in evoked as compared to spontaneous activity seen during waking may serve to heighten sensitivity to visual stimuli during the waking state. It should be added that in spite of its occurrence in the absence of external stimulation, spontaneous activity undoubtedly has a variety of functions, as pointed out by Bremer (12) and Granit (25). The analogy between the ratio of evoked to spontaneous activity and the ratio of signal to noise is thus intended to be entirely descriptive.

It has been mentioned that during waking 17 of 90 units showed arrest or virtual arrest of spontaneous discharge. Arrest of activity such as this has been described by Hubel (28) and Jasper (33) and has been investigated in acute preparations by Whitlock *et al.* (58). The latter authors found that discharge of units in motor cortex was arrested during EEG arousal induced by high frequency stimulation of the thalamic reticular formation. Such stimulation also eliminated the high frequency spike discharges which followed application of dilute strychnine to the cortex, but failed to block the discharges induced by concentrated solutions of strychnine. These authors suggested that a possible explanation for the arrest of spontaneous activity seen in these experiments was active inhibition of neuronal elements in motor cortex during arousal.

In the present experiments there is little evidence available as a basis for deciding the sites and mechanisms responsible for reduction of spontaneous discharge in those cases in which it occurred during waking. Equally unexplained are the increases in the rate of spontaneous discharge which occurred in 42 of 90 units studied. It is possible that reductions in spontaneous discharge may reflect changes in active inhibitory processes at a cortical level, alterations in activity in the lateral geniculate, or even in the retina. The observations of Granit (26) and Dodt (17) indicate a possible mechanism for alterations of retinal activity during arousal. Such alterations might be reflected in corresponding changes at a cortical level. Jung's (38) demonstration of the influence of the diffuse thalamic projection systems on activity in the visual cortex indicates another possible origin for some of the effects we have observed. Finally, there is much evidence that alteration of activity in the mesencephalic reticular formation (43, 44, 49) is an important determinant of the alterations in cortical activity associated with sleep and arousal. There are, of course, a number of additional regions of the brain which may be involved in mediating alterations of visual activity. The present investigations cast little light on which of these mechanisms may be involved in the effects which have been described in this report.

In the section on methods it was pointed out that the observations which have been presented were carried out in darkness. Extraneous visual stimulation was eliminated in an attempt to dissociate the effects of waking, per se, from the effect of increased visual input which is ordinarily associated with waking. Unfortunately, equalization of sensory input during waking and sleep was achieved only for the visual modality. During waking the cats often moved. The result was necessarily an increase of cutaneous, proprioceptive, and auditory stimuli, all generated by the cat itself. The studies of Buser and Borenstein (14), and Jung (38) have shown that such nonvisual stimuli influence neuronal discharge in the primary visual system. It seems probable, therefore, that the results obtained in the present studies reflect more than 'pure waking.'

The importance of the behavior associated with sleep or waking in determining patterns of neuronal discharge in these two states has been strikingly indicated in several experiments. For the sensorimotor cortex of the monkey, for instance, Jasper (33) has found that the amount of neuronal discharge is markedly increased during movement. A similar relationship between eye movements and neuronal discharge in the superior colliculus may be seen during sleep. Huttenlocher (personal communication) has made simultaneous recordings of eye movements and unit discharge in the superior colliculus of the sleeping cat. Eye movements (which commonly occur during sleep without EEG slowing) are associated with striking increases in rate of discharge by many units in this region during sleep. These observations make it clear that findings which are obtained in studies of neuronal activity during sleep and waking will depend to a very large extent on the associated behavior of the experimental animal.

SUMMARY

Waking is associated with a reduction of spontaneous discharge rate in a majority of cortical units, and with a prolongation of the suppression of spontaneous discharge by a single lateral geniculate stimulus. In association with reduction of spontaneous discharge there is often a reduction of discharge evoked either by electrical stimulation of ipsilateral lateral geniculate radiations or by diffuse illumination of the contralateral retina. In many units in which activity is evoked, waking is associated with an increase in the ratio of evoked to spontaneous discharge. The increase of this ratio during waking may serve to heighten sensitivity during the alert state.

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Behavioral changes associated with changes in concentrations of brain serotonin

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OUR INTEREST IN THE TECHNICS REPORTED HERE had its inception in the early 1940's when Dr. Harold Himwich was working actively with one of us (WAH) in the field of cerebral metabolic rate (CMR) in man (14) and in dog (15). At that time it was felt that very interesting and important contributions could be obtained if a suitable technique could be developed for the *in vivo* study of regional metabolic rates in the brain. Several years ago our interest in this field was revived by Dr. Percival Bailey, who remarked that in the dog the clipping of the basilar artery would separate effectively the vertebral and carotid blood entering the brain. From this point the attempts to divide the arterial supply of blood to selected brain regions of the dog has become one of the problems of the Thudichum Psychiatric Research Laboratory, and we feel that we have made some progress toward the solution of this problem. We have not yet attempted the isolation of venous blood arising from isolated brain regions that would be necessary for regional CMR studies. At this time we wish to present a progress report of the isolation of arterial blood supply to the brain and of the attempts made to define the limits of error and the practical utility of this technic in fields other than CMR.

ANATOMICAL CONSIDERATIONS

In the dog the majority of blood reaches the brain through four large vessels: the two vertebral arteries which join to form the basilar artery, and the two common carotid arteries which divide first into the internal and external carotid, the latter giving rise to the internal maxillary which joins the internal carotid through the anastomotic artery. The blood from these two sources enters the meninges and the circle of Willis as the internal carotid (16). At the level of the origin of the internal carotid, that is, in the region of the carotid bulb, are found two small arteries which may anastomose with the vertebral. The anastomoses between the occipital artery and the vertebral occur under the wing of the atlas; possible anastomoses of the ascending pharyngeal have been less thoroughly described. Thus, in order to avoid any possible contamination of vertebral blood by carotid blood all vessels originating from the common carotid with the exception of the internal and the external carotid must be ligated.

In the dog the distribution of blood in the circle of Willis has been described by Jewell and Verney (17). We have performed more fragmentary studies than they but find that our results are in agreement with their data. The blood entering from the internal carotid tends to remain segregated on the ipsilateral side of the hemisphere; that is, the left anterior cerebral and the left middle cerebral are filled by blood from the left internal carotid and vice versa. A pronounced streamlining was found in the basilar artery of rabbit (19) and man (22) so that blood from the left vertebral passes into the left side of the circle of Willis with little or no mixing with blood from the opposite vertebral. Jewell and Verney (17) have confirmed this segregation of blood flow in the dog. These authors find that a limited mixing of carotid and vertebral blood occurs at the trifurcation of the internal carotid inside the calvarium (fig. 1). If the basilar artery is clipped the vertebral blood can reach the clip but will not contribute to the blood of the circle of Willis. In these circumstances the circle will be filled entirely by carotid blood which will pass down in the basilar as far caudad as the clip (fig. 2). In the same manner, if a clip or a ligature is placed at any point on the circle the pressure will equilibrate throughout the circle and blood will reach a uniform distribution in the entire area. This uniformity is facilitated by the fact that in this arterial circle there are neither valves nor structures directing the normal flow of blood.

METHODS

In order to inject into arterial blood going to selected parts of the brain circulation in the conscious and unrestrained dog it was necessary to have access to the carotid and the vertebral circulations without opening the skin. To accomplish this purpose we have used either chronically implanted catheters (6) or skin pedicles (12).

Chronically implanted catheters. According to our experience such catheters could be kept open and in good working order for approximately 2 weeks; after this time for a variety of technical reasons they were not usable. To have exclusive access to the carotid circulation we ligated the lingual artery and any arteries other than the internal and external carotids arising in the area of the bifurcation of the carotid. The catheter was usually

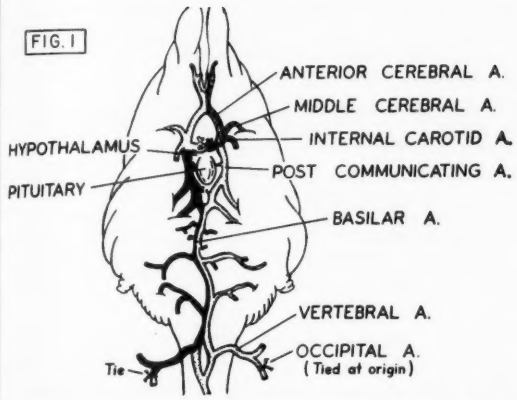

FIG. 1. Normal blood distribution in circle of Willis (based on Jewell and Verney (17).

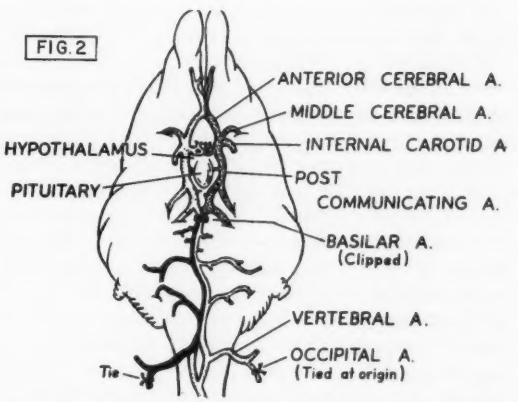

FIG. 2. Effect of clip on basilar artery on blood distribution in circle of Willis.

inserted through a branch of the thyroidal artery although in our earliest experiments the lingual was also used. In these studies we had to choose between diffusion of the injected material into the face of the animal if the external carotids were left open or of disturbing seriously the hemodynamics in the circle of Willis on that side, if we ligated the external carotid. Since the internal maxillary carries approximately ⅔ of the blood (Dr. George Christensen, personal communication) which enters the brain at the internal carotid, we have preferred to lose some of the material by distributing it in the face rather than cause alterations in the hemodynamics of the circle.

In gaining access to the vertebral circulation a number of other problems were encountered. The main omocervical trunk and the axillary artery were ligated. The internal thoracic and the costacervical were generally not ligated. The catheter was inserted through a branch of the omocervical so that its tip lay close to the origin of the vertebral artery. The catheter was always implanted on the left side, since in the dog both common carotids arise from the right subclavian (20), and if a catheter were put on that side there would always be the possibility of some material being carried into one or both carotid arteries. The position of the catheter was checked by x-rays following the injection of radiopaque material, and in each dog at the time of autopsy.

Skin pedicles. The exteriorization of the common carotid is relatively simple. It was first described by Van Leersom (23) and was used by Goldblatt (8) in his classical experiments on hypertension in the dog. To form carotid skin pedicles the bifurcation of the common carotid is prepared in the same manner as when chronic catheters are implanted (see preceding paragraph). The common carotid artery is freed of the surrounding tissue, brought to the surface and enclosed by a strip of skin, to make a skin pedicle or a 'carotid loop.' This procedure places the artery immediately under the skin where it is readily accessible for the withdrawal of blood or the injection of solutions. The problems encountered for the vertebral route were quite different from those involved in the exteriorization of the carotid artery. The distance between the origin of the vertebral artery from the subclavian and its entrance into the sixth cervical vertebrae (20) is very short. Jewell and Verney (17) in considering this problem suggested that the artery might be freed from the transverse processes of the cervical vertebrae for a sufficient length to allow the formation of a loop. In our hands such a procedure involved considerable bleeding from the vertebral sinuses and we finally adopted a different technique. We made use of a length of the common carotid on the same side in order to acquire enough flexibility to form a 'vertebral loop.' The common carotid freed of adjacent tissue was tied and cut one cm distal to the carotid bulb. The vertebral was tied and cut as close as possible to its origin from the subclavian. The distal end of the carotid was anastomosed to the proximal end of the vertebral. This operation gave us a length of carotid artery which could be effectively exteriorized and covered with a skin flap. From a physiological standpoint the procedure is not ideal and many criticisms and questions must still be answered. The carotid artery normally carries more blood and is subject to a greater head of pressure than is the vertebral artery. We are therefore pouring a greater volume of blood under increased pressure into the vertebral artery than it normally carries. The consequences of this unphysiological situation are still unknown. We have in many instances placed a clip on the posterior communicating artery on the same side as the vertebral loop in order to limit the effect of increased pressure on the distribution of the vertebral circulation of the circle of Willis on that side. It is probable that the increases in pressure and volume are dissipated through the many anastomoses between the vertebral and the omocervical artery in the neck. Dye studies which we will refer to later suggest that this may be the case especially in animals who have had an exteriorized vertebral loop for over a year.

Distribution of solutions injected in skin pedicles. The theoretical possibilities of neurosurgical tampering with the distribution of blood in the circle of Willis and of exteriorizing the carotid and vertebral arteries have been analyzed in the preceding paragraphs. Five different technics were used to investigate the area of distribution of solutions injected intra-arterially into skin pedicles.

1) Arteriography was used to study the distribution of radiopaque material injected into exteriorized arteries afferent to the brain. The amount of information derived from this method is of course limited since the radiopaque material must be injected quickly and under great pressure. With this technique the patency of the vessels involved and the maximum filling which can be expected when a drug is injected by the same route can be demonstrated. Arteriography revealed that a clip on the basilar or anywhere on the circle of Willis probably forms a cul-de-sac, into which the injected material even under pressure does not penetrate readily. Such areas however must have a relatively active metabolic exchange via the blood stream since the animals do not show any gross neurological deficit which could be ascribed to areas of poor circulation. Examples of the x-rays obtained have been published elsewhere (12).

2) A constant volume of Monastral fast blue (supplied through the courtesy of Imperial Chemical Industries Ltd.) was injected into either a carotid or a vertebral loop with an infusion pump set for a constant rate. These terminal experiments were performed with the chest open and the animal under artificial respiration. At the moment in which the injection was terminated the ascending aorta was clamped to stop the blood flow. The unopened calvarium with adjacent tissues was then placed in the fixative recommended by Jewell and Verney (17) and the tissue gradually removed until the brain was free. The brain was embedded in a mixture of cornmeal and gelatin and sliced 3 mm thick on a meat-slicing machine. The details of this technique as well as the results obtained with all animals used in the behavioral experiments will be published *in extenso.* It is sufficient to mention here that the clipping of the basilar artery at the mid-medullary level always prevented dye injected in a carotid loop from reaching the entire medulla oblongata. Conversely, in such animals with the basilar clipped, the dye injected in a vertebral loop never reached the midbrain tegmentum.

3) To study changes in the dynamics of the circle of Willis resulting from the clipping of the basilar artery, blood pressure was simultaneously recorded from the two lingual arteries. The blood pressure in the linguals fell progressively as the two common carotids and the two vertebral arteries were clamped in sequence. In dogs with the basilar artery clipped, the clamping of the vertebral arteries was without any influence upon the pressure in the lingual arteries. These results (1) demonstrate that a clip on the basilar artery effectively separates the carotid from the vertebral circulation.

4) The area of distribution of an intracarotidly injected solution was studied by performing analyses of

TABLE 1. *Serotonin Content of Paired Brain Structures*

Dog Number	Caudate Nucleus		Hippocampus		Thalamus	
	Loop-side, γ/gm	Other, γ/gm	Loop-side, γ/gm	Other, γ/gm	Loop-side, γ/gm	Other, γ/gm
1	1.716	0.937	0.420	0.487	0.591	0.300
2	4.333	2.750	0.966	0.675	0.987	0.258
	Loop side higher				No difference	
	Occipital cortex				Pyriform cortex	
	Caudate nucleus				Frontal cortex	
	Thalamus				Cingulate cortex	

the serotonin contents of paired brain structures of dogs pretreated with tranylcypromine (2 mg/kg i.v. 6 hr before) and injected with 5-hydroxytryptophan, 5 mg/kg, into an exteriorized carotid artery. The results (table 1) show very clearly that the increase of serotonin concentration was greater in the loop side than in the opposite side for certain brain structures. For instance, the caudate nucleus and the thalamus show a marked increase on the side of the loop, whereas in the hippocampus this difference is not consistent in the different animals. This observation is of interest since the hippocampus probably received most of its blood supply from the vertebral (17).

5) Distribution of drugs injected into the previously divided arterial blood supply to the brain was also used to evaluate the area of distribution of drug injected by a study of the pharmacological results elicited. In the same animal the gross behavioral effects caused by drugs injected by one or more arterial routes were compared to the results of intravenous injections. The drugs used were bufotenin and 5-hydroxytryptophan. The latter was given only after pretreatment with a monoamine oxidase inhibitor. These results are described in detail below.

Behavioral results with bufotenin. A constant dose of 1 mg/kg was injected into a chronically catheterized artery or intravenously in 12 dogs (6). In a few animals we were able to compare the effects of bufotenin injected intravenously with those obtained when this drug was given to the same dog in both the carotid and the vertebral arteries. The catheters were implanted usually one at a time and the dogs allowed 48 hours to recover from the surgery before the studies were made. To minimize interference with cerebral hemodynamics the catheters were made of very fine polyethylene (PE-10) and were at least 18 inches long. The dose of bufotenin (mono or di-oxalate) required was dissolved in 2 cc of water buffered to pH 7.4. Using the intravenous or vertebral routes the pharmacological response was immediate. Sometimes the effects appeared before the injection was completed. With different latencies of development and independent of the route of injection, the dogs exhibited various degrees of muscular weakness (19) similar to that described by Fabing (7) as reversible paraplegia. The severity of this symptom was variable. In the extreme cases the dogs could not or would not

TABLE 2. *Percentage of Animals* Showing Marked Response to 1 mg/kg Bufotenin*

Symptoms	Route of Injection		
	I.V. %	Carotid %	Vertebral %
Muscular weakness	92	82	82
Salivation	27	22	82
Howling	18	22	64
Crying	36	22	45
Hyperesthesia	55	30	91
Fear	18	44	82
Dissociated behavior	18	11	55

* 9–12 animals injected by each route.

stand. Irrespective of where the intravenous injection was made the hind legs were the first to show the symptoms. In any given dog the symptoms following intravenous injection were greater than those obtained when the drug was given via the carotid route (table 2). Also in any individual dog the most severe symptoms were observed when the injection was given into the vertebral catheter. Some dogs receiving bufotenin intravertebrally howled for better than 4 hours, appeared insensitive to visual or auditory stimulation, but were hypersensitive to touch. They often cried or howled if they were touched without attempting to defend themselves. Clipping of the basilar artery made no change in the symptoms caused by injection of bufotenin into the vertebral artery. This observation made on dogs in which the drug passed cephalad only as far as the clip led us to suggest that the bulbar portion of the reticular formation or other structures in the medulla might be the site of action of bufotenin injected into vertebral catheters (11).

In other experiments the effects of bufotenin injected into catheters placed in the vertebral artery, carotid artery or the aorta at the level of the origin of the phrenico-abdominal artery were compared with intravenous administration. Injection into the aortic catheter resulted in maximal hypertensive responses, greater than those caused with any other route of injection. The marked pressor response when the drug was delivered to the adrenals was probably due to the release of catecholamines from the adrenal medulla. However, the behavioral response to bufotenin injected by this route was no greater than that following the intravenous administration (11). Thus the peripheral sympathomimetic effects elicited by this psychomimetic drug might be ruled out as determining factors in the behavioral responses to bufotenin.

Behavioral results with 5-hydroxytryptophan. A clear indication of the site of action of drugs in CNS can be achieved by a demonstration of the accumulation of the drug or its metabolites in selected structures of the brain. Technical difficulties impair the use of bufotenin in this regard but do not limit that of 5-hydroxytryptophan (5-HTP). In the latter case serotonin formed by decarboxylation of the biological precursor (5-HTP) can be quantitatively determined. The dogs were pretreated with 2 mg/kg of tranylcypromine, a monoamine oxidase inhibitor (MAOI) (18), exactly 6 hours before the injection of 5-hydroxytryptophan to avoid interferences by monoamine oxidase (MAO) activity which could defeat our purpose by destroying serotonin as fast as it was formed. We would like to emphasize that the sequence of symptoms as described below is strictly dependent upon the experimental situation as defined by doses of drugs, routes of injections and time lapses between injections. If some of these conditions were altered the symptoms might either telescope or spread out to such an extent as to make the identification of the syndrome difficult. The results induced with 5-hydroxytryptophan and a MAOI under our circumstances are quite clear-cut (13). The syndrome can be summarized as follows: the animal will first show a transient drowsiness exemplified by a tendency to curl up in a corner of the cage and pay little or no attention to what is going on in the room (10–15 min. after the injection). This sedation is usually followed by a marked extension of the toes of the fore feet and later of the whole forelegs (20–30 min. after the injection). At this time, or slightly later, the toes of the hind feet and the hind legs also begin to show hyperatonic extension. Face licking and grimacing appear intermittently at this stage. Hyperthermia and panting may develop 30–40 minutes after injection, and more frequently with high doses of 5-HTP. Occasionally we have had panting without hyperthermia, similar therefore to the so-called cortical panting (9). The dogs next become more active and spend their time walking briskly around the area in which they are confined (40–50 min. after the injection). They appear to be at first quite alert to stimuli from the environment around them, but their locomotion becomes rapidly impaired by incoordination accompanied by a hunched back. This motor disturbance progresses gradually into a steppage gait, possibly resulting from a retardation in the starting phase of locomotion associated with dysmetria. As the syndrome unfolds the animals mark time with their hind feet and are forced to increased motor activity. The hind legs show a blind festernation and even when they try to sit their hind legs exhibit rhythmical and apparently involuntary movements. Ataxia and dysmetric movements become more and more pronounced and when walking the dogs tend to keep a part of their body near the wall. The syndrome gradually develops into a stage which can be called obstinate progression. This is characterized by constant and apparently aimless walking

TABLE 3. *Development of Symptoms in the 5-HTP Syndrome*

Behavior	Motor	Other
I. Transient drowsiness	A. Extension toes + paws	Salivation
	B. Incoordination, hunched back	Panting
	C. Steppage	Hyperthermia
II. Aggression	III. Hyperactivity D. and obstinate progression	

DOSE 5-HTP AFTER MAOI[1]	3mg/kg	4mg/kg	5mg/kg	
I. I. V. CAROTID	--- ---	--- ---	3+ 1-2+	NO INTERFERENCE WITH BLOOD DISTRIBUTION
A. I. V. VERTEBRAL	--- ---	2+ 3-4+		

1 TRANYLCYPROMINE 2mg/kg, 6 HRS. BEFORE
1+ = EXTENSION
2+ = MOTOR INCOORDINATION
3+ = STEPPAGE GAIT
4+ = OBSTINATE PROGRESSION, HOSTILITY

FIG. 3

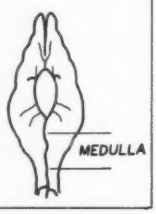

FIG. 3. Response to 5-HTP injected by various routes with normal blood distribution in the circle of Willis.

DOSE 5-HTP AFTER MAOI[1]	3mg/kg	4mg/kg	5mg/kg	
B. I. V. CAROTID	--- ---	1+ ---	3+ 4+	BASILAR CLIPPED DRUG REACHES BRAIN CEPHALAD TO CLIP
A-1. I. V. VERTEBRAL	--- ---	3+ 1+		DRUG REACHES BRAIN CAUDAD TO CLIP

1 TRANYLCYPROMINE 2mg/kg, 6 HRS. BEFORE
1+ = EXTENSION
2+ = MOTOR INCOORDINATION
3+ = STEPPAGE GAIT
4+ = OBSTINATE PROGRESSION, HOSTILITY

FIG. 4

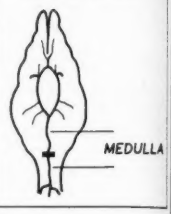

FIG. 4. Response to 5-HTP injected by various routes when basilar artery is clipped.

until the dog finds a corner or a support on which to push obstinately until exhausted. During the obstinate progression the dogs are no longer sensitive to light, sound or touch. This condition may endure for as long as 3–4 hours and is followed by spontaneous recovery. Hostility and aggression both toward the handlers and other dogs may precede the obstinate progression. We have arbitrarily divided these symptoms into two groups—the postural and the behavioral syndromes (table 3). The severity of the behavioral aggression is often divorced from the postural syndrome and is independent of the characteristic attitudes of the individual dog toward his environment. We feel that the obstinate progression is probably a combination of both motor and behavioral components. In contrast to the symptoms resulting from bufotenin injection those due to 5-HTP develop slowly and it may take an hour for a dog to begin to show marked motor incoordination. The rate at which the symptoms appear seems to be more an individual matter than it is a matter of dosage. Larger doses of 5-hydroxytryptophan seem merely to prolong the symptoms, and beyond a certain point do not accelerate their development nor increase their severity. Salivation, hyperthermia and panting may accompany the syndrome described above. We observed rectal temperatures of 112°F, but no positive correlation could be made between hyperthermia and the severity of symptoms. Comparable effects were reproduced by the same dose of the amino acid repeated after 7 days, indicating no adaptation to the drug.

Bogdanski and his colleagues (2) have described the symptoms occurring in dogs given up to 60 mg/kg of 5-hydroxytryptophan without pretreatment with a monoamine oxidase inhibitor. In our experience in a few male dogs given 40 mg/kg of 5-hydroxytryptophan, but not pretreated with MAOI, obstinate progression may be associated with spontaneously occurring orgasms. Orgasms were never seen in dogs given doses of tranylcypromine larger than 2 mg/kg followed by 6–7 mg/kg of 5-hydroxytryptophan. In the latter experimental situation the sequence of events was eventually similar to that caused by bufotenin, insofar as these dogs became paraplegic.

Four typical examples of individual dogs receiving 5-HTP through different routes will be given here. The symptomatology has been arbitrarily rated from 1 to 4+. In two of these dogs there was no artificially induced interference with distribution of blood in the brain (fig. 3). The injection of 5 mg/kg of 5-HTP intravenously in one dog (*I*) produced a 3+ syndrome, whereas the injection of the same dose into the carotid loop of the same animal resulted only in extension of the toes and legs and some evidence of motor incoordination (1+ syndrome). Another dog (*A*) exhibited a mild symptomatology in response to 4 mg/kg i.v. of 5-HTP and when the intravertebral route of injection was used, a 4+ syndrome ensued. These behavioral data suggest that the brain area which normally receives blood from the vertebral artery is more sensitive to the drug than are those areas which receive blood from the carotid.

A more precise localization of the action of this drug was attempted in dogs with the basilar artery clipped at the midmedullary level (12). In *dog B* 5 mg/kg i.v. resulted in a 3+ symptomatology, that is, extension, motor incoordination and a steppage gait (fig. 4). The same dose given in the carotid loop elicited a full constellation of symptoms including obstinate progression and hostility. In this case we must remember that the injection into the carotid loop reached the entire area of the brain cephalad to the clip. A second dog (*A-1*) showed steppage gait as well as motor incoordination and extension in response to 4 mg/kg of 5-hydroxytryptophan intravenously. The same dose given into the vertebral loop of *dog A-1* caused only drowsiness and extension of the paws. In this case the drug reached the brain only caudad to the clip. Comparison of the results from these two groups of dogs suggest that the major site of drug action lies some place between the midmedullary level and the area of the brain normally irrigated by carotid blood. Reports of Hess *et al.* (10), that in rats given 5-HTP the peak of the serotonin

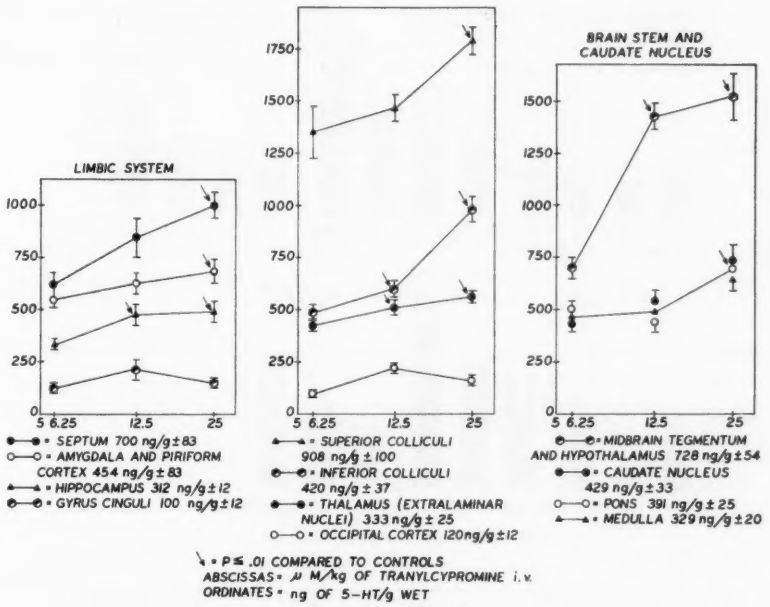
LIMBIC SYSTEM
BRAIN STEM AND CAUDATE NUCLEUS
SEPTUM 700 ng/g ± 83
AMYGDALA AND PIRIFORM CORTEX 454 ng/g ± 83
HIPPOCAMPUS 312 ng/g ± 12
GYRUS CINGULI 100 ng/g ± 12
SUPERIOR COLLICULI 908 ng/g ± 100
INFERIOR COLLICULI 420 ng/g ± 37
THALAMUS (EXTRALAMINAR NUCLEI) 333 ng/g ± 25
OCCIPITAL CORTEX 120 ng/g ± 12
MIDBRAIN TEGMENTUM AND HYPOTHALAMUS 728 ng/g ± 54
CAUDATE NUCLEUS 429 ng/g ± 33
PONS 391 ng/g ± 25
MEDULLA 329 ng/g ± 20
* P ≤ .01 COMPARED TO CONTROLS
ABSCISSAS = μ M/kg OF TRANYLCYPROMINE i.v.
ORDINATES = ng OF 5-HT/g WET

FIG. 5. The serotonin content of various parts of dog brain in relation to the dose of tranylcypromine.

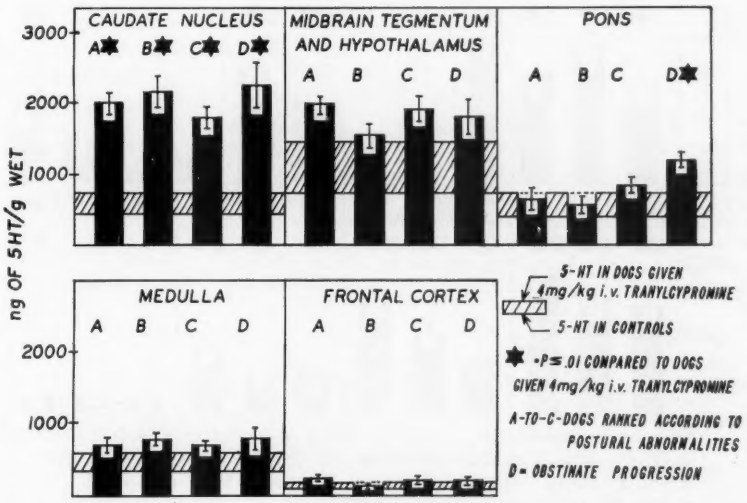
CAUDATE NUCLEUS
MIDBRAIN TEGMENTUM AND HYPOTHALAMUS
PONS
MEDULLA
FRONTAL CORTEX
ng OF 5HT/g WET
5-HT IN DOGS GIVEN 4mg/kg i.v. TRANYLCYPROMINE
5-HT IN CONTROLS
* P ≤ .01 COMPARED TO DOGS GIVEN 4mg/kg i.v. TRANYLCYPROMINE
A-TO-C-DOGS RANKED ACCORDING TO POSTURAL ABNORMALITIES
D = OBSTINATE PROGRESSION

FIG. 6. 5 HT content of brain parts in dogs given 5-HTP (5–7 mg/kg i.v.) 6 hr. after tranylcypromine (2 mg/kg i.v.).

(5 HT) increase in the whole brain does not correlate with the peak of pharmacological effects, may indicate that the symptoms caused by 5-HTP are not related to the 5 HT increases. We have analyzed this problem further to correlate discrete structural increases of 5 HT to the severity of symptoms caused by 5-HTP.

Brain serotonin and the 5-HTP-induced syndrome. A bioassay procedure, previously described (5), was used to determine serotonin (5 HT) in brain parts of dogs. Brain dissections were performed after perfusion of the carotid arteries with saline. The 5 HT content of brain structures of dogs given increasing intravenous doses of tranylcypromine 6 hours before death increased characteristically with the dose of MAOI (fig. 5). The occipital and the cingulate cortex never contained amounts of 5 HT greater than in the controls ($P < .01$). On the other hand the serotonin content of hippocampus, inferior colliculi, thalamus (extralaminar nuclei) and midbrain tegmentum-hypothalamus area was increased in dogs given 2 mg/kg (12.5 μM) of tranylcypromine. Greater doses of the inhibitor (4 mg/kg) were required in order to obtain a similar effect in amygdala, piriform cortex, septum, superior colliculi, caudate nucleus, pons and medulla.

Pscheidt and Morpurgo (21) have analyzed the brains of dogs receiving 4 mg/kg of tranylcypromine for norepinephrine using both a chemical and bio-assay method. The chemical method, in agreement with

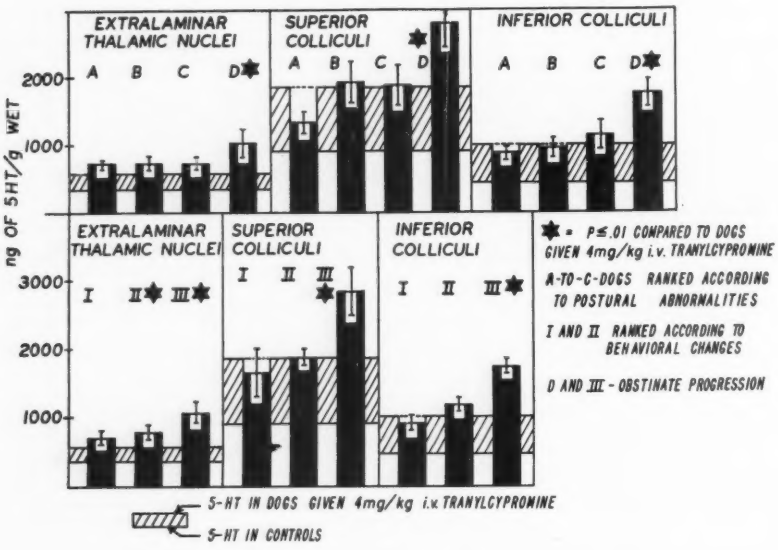
EXTRALAMINAR THALAMIC NUCLEI
SUPERIOR COLLICULI
INFERIOR COLLICULI
A B C D
2000
1000
ng OF 5HT/g WET
3000
I II III
* = P≤.01 COMPARED TO DOGS GIVEN 4mg/kg i.v. TRANYLCYPROMINE
A-TO-C-DOGS RANKED ACCORDING TO POSTURAL ABNORMALITIES
I AND II RANKED ACCORDING TO BEHAVIORAL CHANGES
D AND III - OBSTINATE PROGRESSION
5-HT IN DOGS GIVEN 4mg/kg i.v. TRANYLCYPROMINE
5-HT IN CONTROLS

FIG. 7. 5 HT content of thalamic nuclei, and colliculi in dogs given 5-HTP (5–7 mg/kg i.v.) 6 hr. after tranylcypromine (2 mg/kg i.v.).

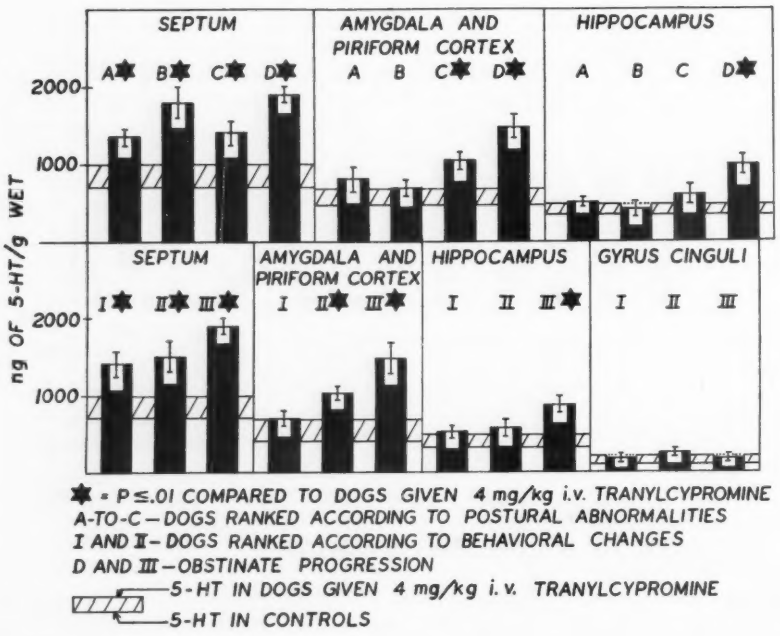
SEPTUM
AMYGDALA AND PIRIFORM CORTEX
HIPPOCAMPUS
GYRUS CINGULI
A B C D
I II III
2000
1000
ng OF 5-HT/g WET
* = P≤.01 COMPARED TO DOGS GIVEN 4 mg/kg i.v. TRANYLCYPROMINE
A-TO-C — DOGS RANKED ACCORDING TO POSTURAL ABNORMALITIES
I AND II — DOGS RANKED ACCORDING TO BEHAVIORAL CHANGES
D AND III — OBSTINATE PROGRESSION
5-HT IN DOGS GIVEN 4 mg/kg i.v. TRANYLCYPROMINE
5-HT IN CONTROLS

FIG. 8. 5 HT content of limbic lobe structures in dogs given 5-HTP (5–7 mg/kg i.v.) 6 hr. after tranylcypromine (2 mg/kg i.v.).

previous reports (4), did not detect any changes of norepinephrine content as a result of MAO inhibition. However, if hypertension and contraction of the nictitating membrane of cats were used to assay for catecholamines, the area of the midbrain tegmentum-hypothalamus of dogs given tranylcypromine was found to contain increased amounts of an unidentified norepinephrine-like substance.

Since neither behavior nor posture of dogs given 25 μM/kg (4 mg/kg) tranylcypromine differed from that of the controls, the 5 HT levels of brain parts of these dogs were used as a basal line (fig. 6) to study correlation between serotonin increases and severity of symptoms induced by 5-HTP under our conditions. Four groups of four dogs, each animal killed when he exhibited a definite stage of the 5-HTP-induced syndrome, are illustrated (table 3). The serotonin concentration of brain structures of dogs given 5–7 mg/kg of 5-HTP were ranked according to the severity of the symptoms they exhibit at the time of their death. The greatest serotonin concentrations were found in the caudate nucleus but the amount of this increase did not correlate with the

severity of the symptoms. This effect on the serotonin level was to be expected; this brain structure having a very efficient amino acid decarboxylase system which would actively convert 5-HTP to 5 HT (2). The 5 HT content in the frontal cortex was never significantly greater than that in the control animals or in that of dogs given 4 mg/kg of tranylcypromine. Serotonin concentrations of the midbrain tegmentum-hypothalamus area or the medulla oblongata were greater than in the controls but never significantly greater than in dogs given 4 mg/kg of tranylcypromine. The pons was the only structure (fig. 6) in which the 5 HT concentrations could be correlated with behavior. The serotonin content found in the pons of dogs exhibiting obstinate progression was significantly greater than that of other dogs receiving 5-HTP but killed while showing less severe symptoms. The 5 HT content of the extralaminar thalamic nuclei, superior and inferior colliculi behaved similarly to that of the pons (fig. 7). When serotonin concentrations of brain parts from the same animals were ranked according to the severity of their behavioral disturbances, those dogs exhibiting increased hostility and aggression contained in the extralaminar thalamic nuclei a quantity of serotonin significantly greater than that found in the same area of behaviorally normal dogs given 4 mg/kg of tranylcypromine. When structures of the limbic system (fig. 8) were ranked using a similar criterion the hippocampus like the pons exhibited a significant increase over the basal line only in animals with obstinate progression. The septum like the caudate nucleus was increased significantly in the earliest stage of the syndrome. However, the extent of the increase did not bear a constant relationship to the severity of either neurological or behavioral abnormalities. The 5 HT content of the piriform cortex and the amygdala was greater than the basal line during the III and IV stages of the syndrome, but the cingulate gyrus failed to show the effects of the injected 5-HTP. Moreover, when the dogs were rated according to the degree of their behavioral abnormalities the aggressive animals had a higher 5 HT level in the piriform cortex-amygdala area than was present in the other animals.

These data on the serotonin (5 HT) content of the various brain structures were obtained in a situation in which serotonin has been artificially increased by giving the biological precursor and by inhibiting the destruction of serotonin. They show the correlation between a biochemically induced lesion of increased serotonin in various parts of the brain and the development of behavioral abnormalities accompanied by disturbances of motor integration. Although these data indirectly suggest that amine content is a physiological constant of selected structures they do not furnish information as to the physiological role of serotonin in CNS. By injecting 5-HTP we are forcing the formation of the amine in structures which contain decarboxylase but which do not necessarily depend on 5 HT for their function (2, 3). A comparison between the 5 HT content in the caudate nucleus (figs. 5 and 6) clearly illustrates this point. The extent of changes induced by MAO inhibition, per se, are of a different order of magnitude than those produced by the administration of the precursor of serotonin. Analysis of structures which, like the pons and the medulla oblongata, contain very little decarboxylase suggest that our situation is extremely unphysiological and, therefore, that the syndrome described is the result of a reversible biochemical lesion.

We wish to express our appreciation to Dr. Harold Himwich for his active interest and collaboration in these studies, to Dr. S. L. Goldstein for the neurosurgery and to Dr. R. G. Canham for teaching us the vascular surgery employed in these experiments.

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Motivational effects of brain stimulation and drugs[1]

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THIS PAPER SUMMARIZES our previous work, presents new studies, and illustrates the value of using objective behavioral techniques to study the effects of brain stimulation and of drugs. Since I cannot claim to be an expert in either neurophysiology or pharmacology, the emphasis will be on a behavioral analysis of the physiological effects.

NEED FOR A VARIETY OF MEASURES

My first point is the usefulness of employing a variety of behavioral tests. This point is clearly illustrated by a very simple experiment by Martin Choy in our laboratory.[2] Choy measured the effects of giving via a stomach tube 5 cc of a 2-molar saline solution to rats which were satiated on food and water. He measured the effects at four different intervals after this treatment using tests given on different days in a balanced Latin square design. At each interval he used three different techniques of measurement. The results are shown in figure 1.

You can see that if Choy had used only the measure of rate of bar pressing to work for water on a variable interval schedule of reinforcement, he would have concluded that the treatment had little, if any, effect at the short 15-min. interval. The initial effect on the rate of bar pressing is small and does not approach statistical reliability. However, the other two tests, the amount of quinine required to stop drinking, and the volume of water consumed, do show appreciable and statistically reliable effects at the 15-min. interval. Apparently, the rate of bar pressing to work for water is insensitive at this time interval. Perhaps performance on the bar-pressing test is more easily disrupted, than is the drinking of water, by discomfort from the recently intubated strong saline solution.

Looking now at the latter part of the curves, it is apparent that if Choy had used only the test of the amount of water consumed, he would have concluded that the effect reaches its maximum within 3 hours, since that measure levels off after the 3-hour interval. This conclusion would be false also, since the other two measures show large and reliable rises between the 3 and the 6-hour tests. Perhaps the amount consumed reaches a ceiling determined by the volume of the stomach.

To summarize, in this particular situation the volume consumed is sensitive only at the shorter intervals but seems to reach a ceiling, and the bar-pressing test is sensitive only at the longer intervals. The quinine-deterrence test is reasonably sensitive throughout the range. The value of using more than one test is obvious.

There are at least two major reasons why the results of different tests purporting to measure the same thing may not agree:

1) Most tests are not pure measures of a single variable and hence different ones can be affected differently by variables other than the one we are trying to measure. Our suggestion that the initial discomfort may affect bar-pressing more than drinking is an example. Similarly, drugs are likely to have a variety of side effects which may produce artifacts with certain tests. Some of our present screening tests may be measuring clinically irrelevant side effects which happen to be associated with useful effects in an already known class of compounds, but may not be in the unknowns we are screening.

2) The fact that we have in the English language a word (for example 'hunger' or 'aversive stimulation') to refer to something, does not necessarily mean that the phenomenon is a single unitary variable or controlled by a single center in the brain. The phenomenon may be a heterogeneous cluster of variables. Different tests may be measuring different combinations of these variables, much like the proverbial blind men touching different parts of the elephant, and hence may not always agree. As we try to make sense out of puzzling empirical data, we may find that there are better ways to 'carve nature to the joint' so that we are dealing with purer, more unitary variables. We shall never discover the discrepancies indicating the inadequacies of the 'folk' categories that come down to us in the English language unless we try to compare the results of a number of different tests purporting to measure the same thing.

[1] Work on this paper and on studies cited in it was supported by grants M647 and MY2949 from the National Institute of Mental Health.

[2] Unpublished senior thesis, Yale University, 1956. For descriptions of the tests used see Miller (11).

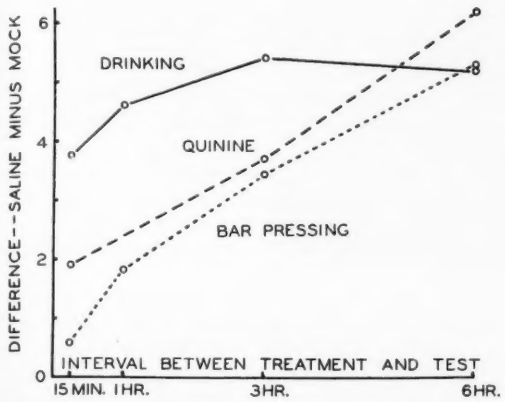

FIG. 1. Different measures of 'thirst' produced by 5 cc of 2 M NaCl solution given to albino rats via a stomach tube. Units on the ordinate are the amounts by which the experimental tests exceeded the control ones given at the same time following a mock injection. To yield measures on various tests, these units should be multiplied as follows: × 1 for quinine score; × 2 for cc of water drunk to satiation; × 10 for bar presses during 9 minutes. Data from unpublished experiment by Martin Choy. Measures described in Miller (11).

'HUNGER' ELICITED BY ELECTRICAL STIMULATION OF BRAIN

Let us now see how a variety of behavioral tests have been used to analyze the effects of electrical and chemical stimulation of the brain.

In his classic experiments Hess (8) showed that electrical stimulation via chronically implanted electrodes in certain areas of the hypothalamus could cause satiated cats to eat. But he presented pictures of a cat stimulated to gnaw on a stick. Therefore, one wonders whether the stimulation elicited a general motivation of hunger or only a specific gnawing reflex. Coons and Miller have conducted a series of experiments to determine whether similar eating elicited by electrically stimulating the hypothalamus of satiated rats is a specific gnawing reflex or has all of the properties of normal hunger. Some of our earlier experiments have been reported elsewhere (10); I want to bring this story up to date.

Localization of stimulus-bound eating effects. Along with Smith (19) and others we have found that eating can be elicited in satiated rats by stimulation via electrodes aimed at the general region of the hypothalamus where Anand and Brobeck (1) found that bilateral destruction would eliminate eating. Stimulation (either unilateral or bilateral) in this region will cause eating which is called 'stimulus-bound' because it starts promptly after the onset and stops promptly after the offset of the electrical stimulation of the brain.[3]

The left side of figure 2 shows the points from which we have elicited such eating. It can be seen that the points are clustered along a line running between the mammillothalamic tract and the fornix. In other words, they are in the general region of Anand and Brobeck's (1) 'feeding area.' The section represented is in the plane of A 5.4 in deGroot's (5) atlas. Points anterior to 5.8 or posterior 5.2 did not elicit eating. To date we have histology on 19 such points that are too far anterior or posterior to elicit eating and are not shown in this diagram.

At the beginning of this work, our technique of enameling the needles left approximately a 0.3 mm bare at the tip. At higher levels of stimulation the eating was disrupted by general excitement and motor activity. Some of our negative cases seemed to be on the brink of eating, when this excitement intervened. Upon being unable to procure more of this particular enamel, we shifted to a new type which left only a minute point at the tip bare. We thought that this smaller area of exposure, allowing a more specific site of stimulation, might aid us in avoiding the general excitement and thus in getting more clear-cut eating behavior. Instead, our results were much poorer. After a good deal of perplexity and dismay, it finally occurred to us to try increasing the bare area at the tip of the needle by scraping off approximately ½ mm of enamel. The yield immediately went up from less than 1 in 10 to approximately 1 out of 3, which was better than with the original enamel. Apparently spreading a relatively mild stimulation over a greater number of units is more likely to yield a good eating response than strongly stimulating a few units.

The right-hand parts of figure 2 show the histology of some of the positive eating points on which we also have additional information. It can be seen that stimulation at some of the points elicited drinking as well as eating. Stimulation of other points elicited biting of a stick as well as eating. Stimulation of yet other points elicited vomiting (supposed to be impossible in the rat) as well as eating. In general the vomiting side effects seem to be elicited from the lower points nearer the ventromedial nucleus, and the drinking from the more medial points.

Figure 3 shows a rat stimulated at such a biting point gnawing on a stick. This brings us back to the question of whether the electrical stimulation of this region in the brain has effects with the general properties of normal hunger or whether it merely elicits a gnawing reflex. How can we find out?

Elicitation of learned food-seeking responses. Normal hunger will elicit not only eating, but also learned food-seeking

[3] The technique of implanting electrodes is described in Miller (11), but we have made the following changes: In order to minimize pulling the assembly out on occasions when rats snag their pins on the hardware cloth of the living cage, we now make the pins out of copper wire (which will bend) and slant them forward at a 45 degree angle. We sometimes connect the indifferent lead to one of the screws in the skull, using #0-80 × ⅛ Fillister Stainless Steel Machine Screws obtained from the Elliott Company, Washington, D. C. To prevent crystallization from sharp bends, we use a blob of dental cement to attach to the pin clutch a short sleeve of plastic tubing over the connecting wire soldered to the pin clutch.

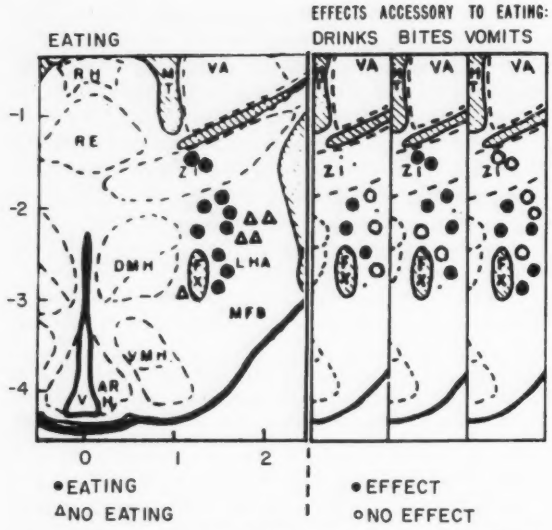

FIG. 2. Points in the hypothalamus at which electrical stimulation elicits eating by satiated rats. Left side shows eating effect; right side shows effects accessory to eating. Section is in the plane of A 5.4 in deGroot (5) atlas.

responses. Therefore we trained rats to press a bar to secure food. Then we put them on a variable-interval schedule so that only at certain unpredictable times did pressing the bar produce a tiny pellet of food. They pressed the bar when food-deprived, but did not when satiated. If the electrical stimulation merely elicited a gnawing reflex, we would not expect it to cause satiated animals to go to the bar and press it, but if the effects of stimulation have the properties of normal hunger, they must motivate the rats to press the bar.

Figure 4 shows a record of a test on a satiated rat. Each bar press moves the pen up a small step; each time a bar press delivers food, a spike is drawn below the cumulative curve. The horizontal line at the beginning of the record and between each test shows that the rat does not press the bar appreciably until he is stimulated. Soon after the brain stimulation is turned on, he starts pressing the bar; soon after it is turned off, he stops.

Control for indiscriminate activation. But perhaps the effects of stimulation are not specific to hunger-motivated behavior after all. Perhaps the stimulation only wakes up the animal and produces a general activation. Since bar pressing is the dominant habit in this situation, that is what he does when activated. How can we test for this?

Coons and Miller selected some of the rats from which stimulation elicited only eating. We trained these rats when thirsty to drink at a water spout, and when hungry to go to a specific place and push back a panel to get the food concealed behind it. Then, when the rats were completely satiated on food but were drinking because they were mildly thirsty, we tested the effects of electrical stimulation of the brain. If the stimulation merely activated the animal, it should cause the dominant activity of drinking to become more vigorous. But if the stimulation had the effects of normal hunger, it should cause the animal to leave the water and go to the food.

Figure 5 shows that the rat did leave the water and go to the food. By repeating this test on a number of animals, we have ruled out the possibility that the effects of stimulation are limited to mere indiscriminate activation.

Response defined by sensory feed-back. Finally, one might revive the gnawing hypothesis for one last fling. If the stimulation does elicit reflex gnawing, it might be unpleasant to try to gnaw with nothing in the mouth so that a satiated rat might prefer to gnaw food rather than the cage or the air. Thus it might perform learned

FIG. 3. Gnawing of stick elicited by stimulating point in hypothalamus which also elicits eating.

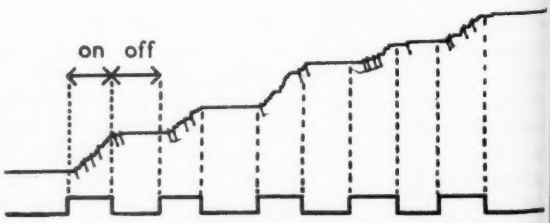

FIG. 4. Food-seeking habit elicited by stimulating satiated rat in 'hunger' area of hypothalamus. Rat had previously learned when hungry to press bar which occasionally delivers food. Each bar press moves pen up a small step. The downward spikes indicate the time when pressing the bar paid off with a pellet of food. The signal marker at the bottom indicates electrical stimulation of the brain. Reproduced from Miller (10).

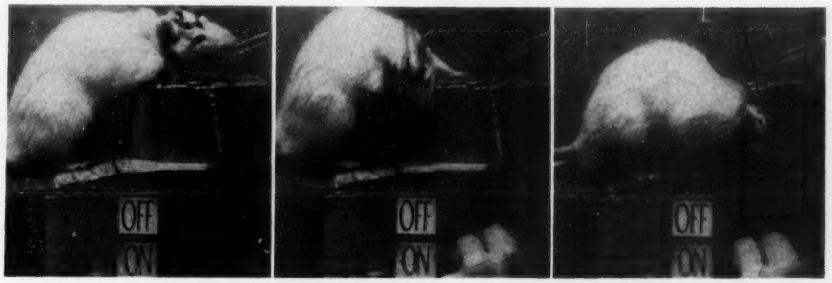

FIG. 5. Electrical stimulation of the brain causes food-satiated but mildly thirsty rat to leave water spout to go to place where he has previously learned that food is hidden.

food-seeking responses. Coons has eliminated this alternative by showing that satiated rats, which will not drink pure water when stimulated, will start to lap up either sugar water or milk as soon as the stimulation is turned on and will stop when it is turned off.

The behavior in these tests shows that the response elicited by the electrical stimulation of the brain is not a specific set of movements, like biting or drinking, but instead is a tendency to respond to a specific taste, that of food. What is the mechanism of a response, such as this, that is elicited by central stimulation but defined by sensory feed-back? We shall return to this question later.

We are also in the process of extending the foregoing type of observation in a somewhat different direction—that of specific hungers. To date we have one rat which, when stimulated, will consistently drink a 4% solution of sucrose but neither pure water nor an 0.8% solution of NaCl. Another rat when stimulated will consistently drink either the sugar or the salt solutions, but not the pure water. It will be interesting to see whether or not such differences can be duplicated and consistently correlated with differences in electrode placement.

Thoroughly satiated rats may be induced by electrical stimulation to eat as much as 20 additional grams of laboratory chow. As they bulge more and more from stuffing themselves, it is not surprising that progressively stronger stimulation is required to elicit eating. Apparently the response is controlled also by sensory feed-back from the stomach (10). The threshold may increase from approximately 8 to approximately 12 ma. Similarly, dextroamphetamine, which seems to reduce or counteract normal hunger, increases the threshold for eating elicited by electrical stimulation of this area of the brain.

Is offset of hunger-inducing stimulation a reward? To recapitulate, we have found that the effect of electrical stimulation of this particular area of the brain has a considerable number of the properties of normal hunger: *a*) it will motivate eating of solid foods or the drinking of liquid ones by a satiated rat; *b*) it will motivate the performance of learned food-seeking habits; *c*) it will cause a mildly thirsty animal to stop drinking water and go to find concealed food; *d*) it is counteracted somewhat by the administration of dextroamphetamine Let us try our luck once more.

To the hungry animal, food serves as a reward to reinforce the learning of the immediately preceding responses. Sufficient quantities of this same food will reduce the hunger to zero. According to a hypothesis, the drive-reduction hypothesis of reinforcement, it is the reduction in the strength of hunger, or in other words 'escape from hunger', that produces the rewarding effect of feeding. According to this hypothesis, turning off the brain stimulation which functions so much like hunger should serve as a reward to produce learning.[4]

Coons and Miller tested this by placing animals in the start box of a simple T maze, turning on the stimulation and leaving it on until they reached a goal box on the correct side of the maze. At first the rats made errors; but they soon learned to run rapidly and directly to the correct side of the T maze. This learning demonstrated that turning off the brain stimulation can serve as a reward, just as does the food which eventually terminates hunger.

Possible mechanism for control via sensory feed-back. Perhaps the drive-reduction notion provides the key to how stimulation can elicit a response which is not specific to any motor movement but instead to a particular sensory feed-back. Other experiments in our laboratory have shown that the taste of food can contribute to reducing the strength of the hunger drive. (10) Perhaps the taste of food reduces the aversive strength of the electrical stimulation of this area of the brain. This might be how the sensory feedback elicits biting food and lapping milk, while not eliciting lapping up of water.

As an aside, it is interesting to speculate that the control by sensory feedback (via a drive-reduction and/or some booster mechanism) evolved first as an innate component of the consummatory response of eating and then later was extended to reinforce the learning of instrumental responses leading to food.

As one would expect from the drive-reduction hypothesis, having food to eat in the T maze causes the rat to postpone running to the place where he escapes the brain stimulation. But perhaps this is solely because picking up the food is incompatible with running. We are planning a more crucial test by slowly injecting sugar water via a fistula directly into the mouth to see whether

[4] For a summary of other studies of hunger relevant to the drive-reduction hypotheses see Miller (10, 12).

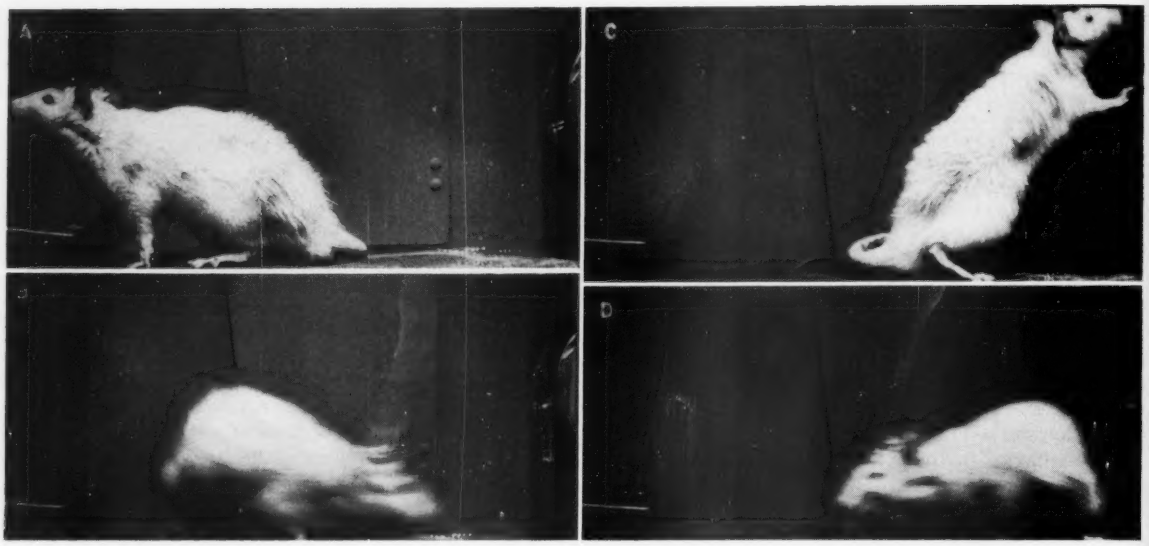

FIG. 6. Rat shows dual reward-aversion effect. First he presses a bar to turn on electrical stimulation in his brain and then rotates a wheel to turn it off.

it will cause rats to tolerate larger amounts of this brain stimulation than they will when pure water is being injected.

Paradoxical reward-aversion effects. The fact that the escape from the hunger-eliciting brain stimulation functions as a reward is a good support for the drive-reduction hypothesis with which my name has been associated (12). It completes a consistent and aesthetically pleasing picture. But further investigation casts doubts on our first simple interpretation of this result.

Since escaping from electrical stimulation in this area of the brain serves as a reward, of course one would not expect the onset of such aversive stimulation also to serve as a reward. We have learned, however, that in exploring the behavioral effects of electrical stimulation of the brain, it is unsafe to leave untested any possibility, no matter how implausible it seems. So we tested the rats to see whether they would press a bar to turn on the stimulation, and we found that they would. In fact they would learn to press one bar to turn the stimulation on and then rotate a wheel (or press another bar) to turn it off.

Figure 6 illustrates such behavior. First the rat approaches and presses the bar to turn the stimulation on. Soon the stimulation becomes aversive, so that he leaves the bar and dashes toward the wheel which he rotates to turn the stimulation off. Then he heads back toward the bar to turn it on, and keeps repeating the cycle. We see that in this region of the brain both the onset and the termination of stimulation are rewarding. The rewarding effect of onset would not be predicted from the strong form of the drive-reduction hypothesis.

But the rewarding effects of both the onset and the termination of stimulation can be secured also from other nearby points where stimulation will not elicit eating. And it can be secured also all along the middle and anterior portions of the medial forebrain bundle. (3)

As an aside I may say that this paradoxical dual effect was first discovered in the vicinity of the posterior hypothalamic nucleus of the cat's brain by Roberts (18) in our laboratory, and that it means that Olds' (16) maps of rewarding and aversive areas of the brain have to be revised because he did not test for the possibility of an aversive effect following a transient rewarding one.[5]

But to return to our main story, the most recent rats on which we have instituted careful tests have shown a threshold for the rewarding effect of stimulation which is slightly, but definitely, above that for the onset of eating. Furthermore, as figure 7 shows, dextroamphetamine (2 mg/kg) raises the threshold for eating as shown by the rise of the solid lines at the central points representing tests 30 minutes after the injection. But the same injection lowers the threshold for bar pressing rewarded by the onset of stimulation.

To recapitulate, we have just seen *a*) that the rewarding effects of onset and termination can be secured from adjacent points which do not elicit eating, *b*) that the thresholds for reward and eating are different, and *c*) that dextroamphetamine has opposite effects on the

[5] Stimulation of the brain in some areas seems to be neutral, in some primarily rewarding, in others first rewarding and then aversive, and yet in others primarily aversive. One may note a suggestive parallel with the hedonic values of various tastes. Thus Pfaffman (17) reports that water is primarily neutral, sugar pleasant, salt pleasant in small concentrations, but aversive in high ones which induce stronger neural firing, and quinine primarily aversive.

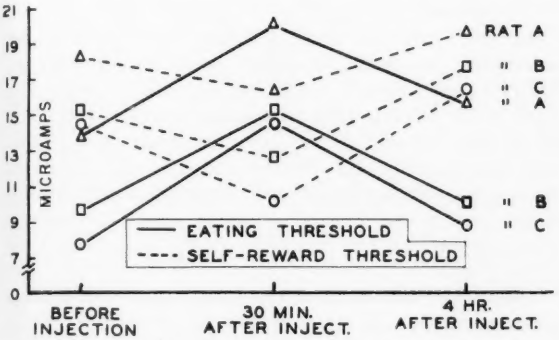

FIG. 7. An injection of dexedrine (2 mg/k) raises the threshold for eating, but lowers it for self reward. Twenty-five tests were given to each rat at each point; the effects are statistically reliable for each of the three rats tested.

thresholds for these two effects. The foregoing facts suggest that the reward and the eating may be independent phenomena which are elicited by the indiscriminate effects of electrical stimulation.

DELAYED EATING FROM POINTS ON PAPEZ' CIRCUIT

The histology on some of our very first rats which ate when stimulated showed an electrode placement very near the mamillo-thalamic tract. In order to test for the possible involvement of this tract we aimed electrodes at the anterior nucleus of the thalamus where this tract terminates. Rats stimulated for a few seconds via these electrodes froze like a living statue during stimulation, then either shuddered or progressively went into a splendid Jacksonian convulsion. After approximately a 4 min. delay, they commenced eating and continued to eat for 2–8 minutes—an impressive after-effect of a brief stimulation. Rats which shuddered would also perform the learned habit of pressing a bar to work for food during the delayed-eating period. But rats that had just recovered from a convulsion acted as though they had temporarily forgotten this habit.

Figure 8 shows the points from which delayed eating has been elicited. These points are scattered rather widely through the brain but seem to be on the classical Papez' circuit.

CONDITIONING OF OR SENSITIZATION TO CONVULSIONS

After being tested for delayed eating daily for a week, some of these animals would go into a complete convulsion, which left them lying on their sides, soon after they were put into the apparatus and before the stimulus was turned on. We are in the process of trying to determine whether this highly atypical behavior is a true conditioned convulsion or merely the result of considerable sensitization, so that the mechanical stress of handling or putting on the electrodes is enough to elicit a convulsion.

Other tests show that these convulsions are aversive. Rats will learn to avoid the side of a T maze where they are regularly given convulsions. This confirms earlier results by Coons and Miller showing that the procedure of administering electroconvulsive shocks through the rat's ears is aversive and hence introduces an artifact into experiments purporting to show a temporal gradient of retrograde amnesia. Such experiments have been used as an argument for a period during which the memory engram is especially susceptible to disruption before it 'hardens'. This is an important problem, but it is being discussed elsewhere (4).

ARREST OF EATING AND OTHER RESPONSES

Opposite effects are secured by extirpating the Anand-Brobeck (1) feeding areas and the nearby region of the ventromedial nuclei of the hypothalamus. The former causes starvation, the latter hyperphagia. Will opposite effects be caused also by stimulating these two areas? We have just seen that stimulating the 'feeding' area elicits eating. Will stimulation of the ventromedial nuclei stop the rat from eating? If so, will it have the other behavioral effects associated with normal satiation?

Smith (19) has reported that stimulation in the region of the ventromedial nuclei does reduce eating. Krasne, in unpublished work from our laboratory, has made similar observations, but his control tests show that the interpretation is not simple. As might be expected from the drive-reduction hypothesis of reinforcement, stimulation in this region has a rewarding effect analogous to that of food which normally reduces hunger. But this rewarding effect is rather weak and brief, passing over quickly into aversion. Rats that will press a bar somewhat hesitantly to turn such stimulation on will rotate a wheel promptly and vigorously to turn it off. It could well be that this aversiveness is what stops the stimulated rat from eating rather than a genuine reduction in hunger. In a series of 10 points studied, Krasne found that a current of 10–30 microamperes is sufficient to

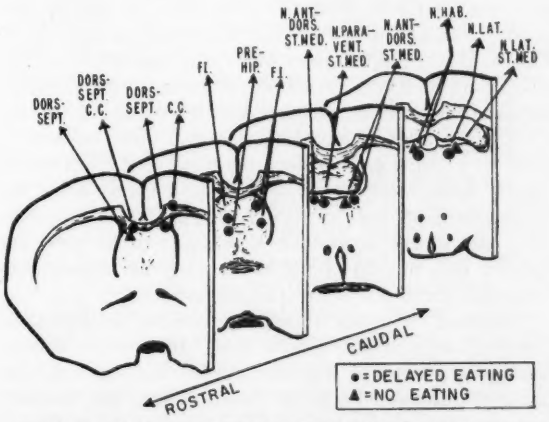

FIG. 8. Points in the rat's brain at which electrical stimulation elicits delayed eating.

XUM

cause the rat to rotate a wheel to turn it off, whereas in each of the 10 comparisons a slightly greater current was required to cause the rat to stop eating. In other words, the threshold for aversiveness was consistently below that for stopping eating.

In additional tests Krasne trained some of his rats to rotate a wheel to escape a very bright light, and possibly also its heat. He found that the stimulation which would stop the rat from eating when hungry would sometimes also disrupt performance motivated in this different way. Thus, it is clear that stimulation of this area involves something more than a specific reduction in the strength of hunger, if indeed it has any effect specific to hunger.

Again we see the importance of not stopping as soon as our hypothesis is confirmed by a single test. If we had done that, we would have mislead ourselves. In this new field of investigation it is essential to design careful behavioral tests to cover all conceivable alternatives.

Finally, Krasne found that there are at least some points in this general area where stimulation can have the immediate effect of stopping the hungry rat from eating and a delayed effect of causing a satiated rat to eat, much like those of Coons' delayed-eating animals which shuddered but did not have a convulsion. Krasne is now trying to design experiments to pin down these effects more quantitatively and to test various interpretations of them.

In the light of the foregoing results, one can see that it is not very meaningful, as is sometimes done, to measure the total effect on food consumption of an hour of intermittent stimulation of a given point in the brain.

ADRENERGIC VS. CHOLINERGIC STIMULATION

Thus far we have been describing experiments on electrical stimulation of the brain. There is reason to believe that the effects of chemical stimulation may be more selective if some way can be found to confine the action to a restricted site. Since injected solutions inevitably tend to spread up the shaft of the cannula used to deliver them, one of my students, Mr. S. P. Grossman (7) has followed MacLean's (9) lead in using minute amounts of crystalline substances. One modified hypodermic needle, stereotaxically implanted in the rat's brain and anchored to the skull with cement, is used as a guide for another modified needle which fits snugly inside and goes down, being stopped when the two squared-off ends are exactly flush. Minute quantities (1–5 μg) of the crystalline substances to be used are tapped into the tip of the smaller needle so that they stimulate the brain when it is inserted.

Grossman (7) has aimed his needles at the same 'feeding' area where Coons and Miller have elicited eating by electrical stimulation. He has worked with satiated animals who never consume food and water in measurable quantities during the prestimulation period.

In one experiment in which 12 satiated rats were tested on each substance in a balanced order with a 3-day rest between tests, Grossman found that the adrenergic substances, epinephrine or norepinephrine, elicited highly significant food consumption beginning from 5 to 10 min. after stimulation and persisting for 20–40 min. An average of 3.0 grams of laboratory chow was eaten under epinephrine and 4.3 under norepinephrine. After eating enough dry food, these animals drank some water, but in other tests, given without any food present, no measurable drinking was elicited by these chemicals.

Quite different effects were elicited by the cholinergic substances, acetylcholine (capped by physostigmine) or by carbachol, introduced via the same cannulae in the same rats. These chemicals did not elicit any significant eating but caused the rats to drink an average of 7.4 cc or 12.8 cc, respectively. The latency and duration of the effect was comparable to that for the eating following adrenergic stimulation.

Injections of NaCl crystals (to control for osmotic stimulation) produced less than one-sixth as much eating or drinking ($P < .02$) as the foregoing substances. Strychnine (to control for general excitation) produced no consumption.

In a preliminary study designed to yield optimal stereotaxic coordinates, Grossman found that very small deviations from the optimal position would completely eliminate the effects. He has also shown that adrenergic or cholinergic stimulation will motivate rats to perform the learned response of pressing a bar to secure food or water, respectively.

At present Grossman is performing further studies to show the generality of these effects by using additional behavioral tests, and to try to control for additional factors such as pH, vasoconstriction, and the possible differential rates of diffusion. Tentatively, his work to date may be taken to indicate: *a*) that the effects of electrical stimulation in the experiments by Coons and Miller probably were not due entirely to stimulating fibers of passage; *b*) that cells or synapses involved in the regulation of both eating and drinking are present in a restricted area in the lateral hypothalamus; and *c*) that the feeding mechanism appears to be selectively activated by adrenergic stimulation while the drinking mechanism appears to respond selectively to cholinergic stimulation.

Grossman's results open up many fascinating lines of investigation. To mention only one of the more behaviorally oriented problems, one could determine whether carbachol will inhibit the eating and food seeking behavior of a normally hungry but water-satiated rat. A positive result would suggest that the inhibiting effects of water deprivation on eating are mediated via a central neural effect of the thirst mechanism on hunger, rather than exclusively via direct effects of bodily dehydration on the hunger mechanism.

Thus far we have been using a combination of behavioral techniques, but we have been using the physiological techniques one at a time. I believe it will be

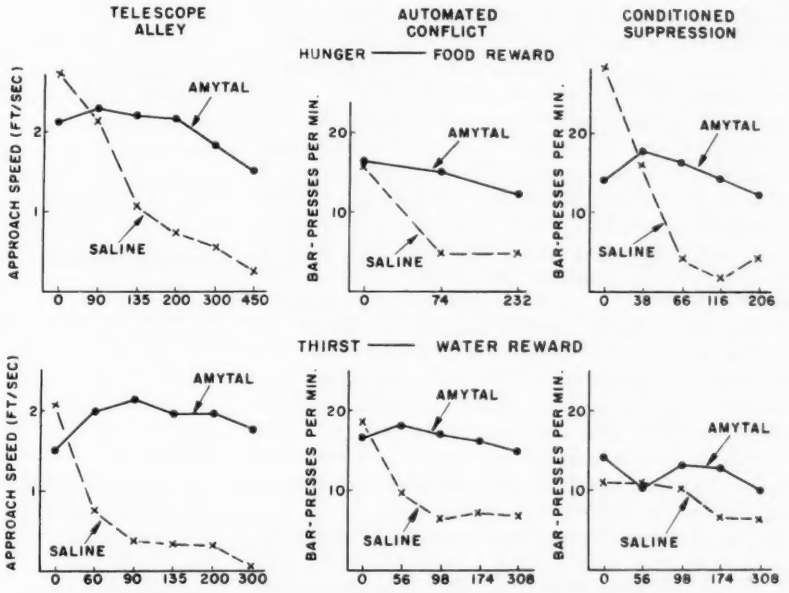
TELESCOPE ALLEY
AUTOMATED CONFLICT
CONDITIONED SUPPRESSION
HUNGER —— FOOD REWARD
APPROACH SPEED (FT/SEC)
BAR-PRESSES PER MIN.
AMYTAL
SALINE
0 90 135 200 300 450
0 74 232
0 38 66 116 206
THIRST —— WATER REWARD
0 60 90 135 200 300
0 56 98 174 308
ABSCISSA OF EACH GRAPH SHOWS THREAT OF SHOCK IN VOLTS
SODIUM AMYTAL 20 mg/kg 20 minutes before test

FIG. 9. Effects of sodium amytal (20 mg/kg 20 min. before) on three different tests of conflict behavior under two different conditions of approach motivation. The abscissa of each graph shows the strength of shock, in volts, to which the fear-eliciting stimuli had been conditioned. No shocks were given during these tests which are described in more detail in Miller (13).

especially fruitful for us to try using analytically various combinations of the physiological techniques—electrical and chemical stimulation, electrical recording, and electrolytic lesions.

PSYCHOPHARMACOLOGY

Finally, our laboratory has also been working on the behavioral effects of drugs. The various techniques, promising problems and certain general strategic considerations have been discussed in a recent review (13).

Part of the work may be illustrated by some recent results on sodium amobarbital. Dollard and Miller (6) have suggested that the effectiveness of this drug as an aid to psychotherapy comes from its ability to reduce the fear-motivated avoidance component of a conflict more than the approach.

Figure 9, summarizing work supervised by Barry and Miller (13), shows the effects of sodium amobarbital on a variety of experimental tests of fear and conflict in the albino rat. In the first trial in the telescope alley test, the animals ran one foot to the reward where they never received electric shock. On each successive trial they were required to run an additional foot and occasionally received shocks which, when they occurred, were stronger the longer the distance to the goal. This test involved considerable movement, the response was running, and the cues for danger were primarily proprioceptive and visual.

In the automated conflict test, the rats pressed a bar for a reward on a variable-interval schedule. The first 2 minutes were safe, but after that an increasingly loud tone signalled unpredictable shocks on the bar which, when they occurred, were increasingly strong the louder the tone. The test chamber limited movement, the response was bar pressing, and the cues were primarily auditory. The conditioned suppression test was similar, except the shock was delivered via the grid floor and was inescapable, so that we were measuring conflict with 'freezing,' rather than with active withdrawal from the bar.

In order to control for any effects specific to the approach drive, the animals in the experiments represented in the top row were motivated by hunger and rewarded by food, while those in the bottom row were motivated by thirst and rewarded by water. It can be seen that the results under all of these various conditions were highly similar. Looking at the beginning of each curve, which represents performance with little or no fear, it can be seen that in general the amobarbital reduced performance below the placebo level. This part of the test acts as a control to show that the effects of the amobarbital were not simply to produce an increase in the approach drive, or to act as a general stimulant.

As the rats encountered cues to which increasingly strong fear had been conditioned, the performance following placebo was markedly reduced. But the performance under sodium amobarbital was not affected nearly as much by the fear-inducing cues. Thus, with fear, performance under the drug was uniformly superior.

The fact that similar results appear in tests involving different cues, different responses and different drives makes it seem less likely that the effects are specific to the peculiarities of a certain testing situation.

One might think that these results are convincing

enough, but other control tests are necessary for an unambiguous interpretation. These additional tests, which are all too often neglected, are in the process of being conducted. For results see Miller (10a).

Control for stimulus change. From other studies (14, 15) we know that changes in the stimulus situation reduce fear-motivated avoidance more than hunger-motivated approach, and thus can produce exactly the kind of effects observed in figure 9. Perhaps the sodium amobarbital does not have an effect specific to the fear mechanism, but only changes the stimulus situation. The test for this possibility is to condition the fear with a second group of animals while they are under the effects of the drug, and then give half of the tests under the same drugged condition and the other half under the new sober one. If the main effect is due to stimulus change, the effects shown in figure 9 should be reversed; but if the drug acts more directly on the mechanism of fear, the results should remain the same. Tests with alcohol show the latter effect. (13)

Control for interference with discrimination. Even though various cues were used in these tests, it is possible that the effect of the drug was not specific to fear, but rather was the general one of breaking down the rat's ability to respond discriminatively to the cues of danger. This possibility is being tested by teaching the rats a different discrimination in which the cues signal nonreward instead of threat of shock. If this discrimination persists under amobarbital, we will know that the drug does not merely interfere with the rat's ability to respond discriminatively to the cues. We shall also know that this drug, like alcohol (13), affects fear differently from the inhibition produced by experimental extinction. However, if we fail to get differential results, we shall have to run an additional test to determine whether the amorbarbital affects the ability to discriminate to cues, or the inhibition produced by the experimental extinction to the negative cue.

Control for primacy of habit. It is also possible that the drug does not have a differential effect specific to fear, but instead has a greater effect on whichever habit was learned more recently. We are testing this hypothesis in two ways. In one experiment we first are training rats to turn right on a T maze, and afterwards training them to turn left by shifting the reward to the other side. A reversion to the original side will indicate that the first-learned habit is more resistant to the effects of the drug. In a second experiment we are attempting to establish the fear of the tone first by pairing it with inescapable shock, and then to train the rat to press the bar during the safe silent period. If the primary effect of the drug is on fear, our results should remain like those in figure 9 for conditioned suppression; if the primary effect is on the more recent habit, the position of the two curves should be reversed.

Transfer of therapeutic effect from drugged to sober state. Finally, if a drug produces a genuine reduction in fear and conflict, it may be useful in protracted mild doses. But it will not be very useful as an aid to re-educating the patient via psychotherapy, unless extinction or counter-conditioning of maladaptive fears will transfer from the drugged to the sober state. Therefore, we are testing to see whether rats induced by amobarbital or other drugs to return to a goal, which had previously been dangerous but now is safe, will transfer their newly practiced courage to the sober state.

I believe that it is highly desirable to perform the foregoing and other kinds of analytical behavioral studies on drugs which have well-established clinical effects. This is one of the ways to build up a science of psychopharmacology which ultimately can provide a rational basis for applications to mental health, analagous to that which organic chemistry provides for the synthesis of new compounds.

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INTERSOCIETY SYMPOSIUM ON GASTROENTEROLOGY

Dynamic interaction among body nutrition, gut mucosal metabolism and morphology and transport across the mucosa[1]

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"The essence of biological and medical problems is that they are enmeshed in a jungle of variables and in the continual flux of the dynamic aspects of living matter. Tests must go on in a milieu with multiple coordinates which may shift dimensions, directions or locations in a bewildering way."—William B. Bean (1).

FEW NORMAL TISSUES manifest as active a metabolism within so dynamic a structure as does the mucous membrane epithelium lining the gastrointestinal tract. The gut lumen contains a brei of biochemical fragments of ingested food split off and bathed in acids, enzymes, bile and bacteria. Together with the mucous membrane, this brei comprises a metabolic interface. Sitting at the very front door of the organism's metabolic machine, this metabolic interface has the job of converting 'food' to 'metabolite' while maintaining an intact 'barrier' to the 'outside world.' Thus, it comprises the first echelon of metabolic adaptability to the organism's environment; it not only responds to the quality and amount of nutrient provided but it conditions the metabolic environment of the tissues behind it. Requiring an abundance of food to feed itself, the gut mucosa promptly reflects nutrient disorders by altered function and morphology. At this level Nutrition and Biochemistry become one with Physiology; and the functional implications of form are uncommonly apparent.

At past meetings of this Federation, isolated fragments of information about gut mucosa have been scattered, with some exceptions, throughout the several societies so widely in time and space that it was thought desirable to initiate an Intersociety Session in the interest of communication and integration.[2]

The form of the gut is rather obviously suited to its large task of absorption. The 'mucous lining' is composed of superficial columnar epithelial cells—only one cell thick. The absorptive surface of the intestine is enlarged greatly by the device of folds and villi. Some estimate of the amount of tissue surface thus afforded can be obtained from figure 1, in which a segment of a rat's small intestine is rolled to form a spool. Five to ten such spools are necessary to accommodate the small intestine alone of a 250 gram rat. Furthermore, electron micrographs reveal that the effective surface is further multiplied many fold by myriad microvilli comprising the brush border of each cell. The beautiful morphologic studies of Palay and others (2, 3) have provided clear demonstrations of the passage of fat globules via the interstices of microvilli into and about the mucosal cells of the rat mucosa. Hogben (4), Wilson (5), Turner (6) and Herbert (7) tell us about the innate intelligence of the gut mucosal cell which permits the selective transfer of some substances while allowing others to diffuse across and rejecting still others.

The thin mucosal surface is kept intact by a busy regenerating apparatus in the gland crypts. Newly divided cells glide along the basement membrane toward the tips of the villi, where, having completed their life cycle, they are exfoliated into the lumen. This vast machine is continuously discarded and regenerated anew every 27–40 hours in the rat and human and somewhat more slowly in the mouse (8–11).

As the anatomy of the tissue reflects its special job, so its biochemical apparatus differs from that of other tissues. Spencer and Knox (12) have summarized what

[1] Supported in part by National Cancer Institute Grants C-2090(C6) and C-4486 and the American Cancer Society.

[2] Dr. Harold L. Stewart and Dr. J. F. A. McManus, of the American Society of Experimental Pathology, whose efforts helped bring about the session, were well aware that 'integration' was something best done by a man's brain; they agreed, nevertheless, that some emphasis on a multidisciplinary viewpoint seemed warranted in the study of this tissue.

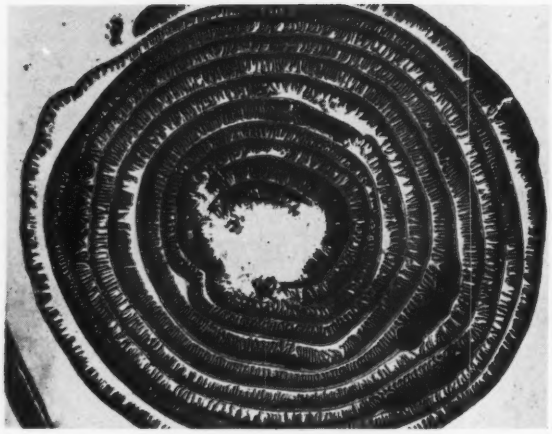


FIG. 1. Spool of opened rat small intestine. × 18.

is known about the gut mucosal enzymes. Until recently, for reasons unclear, the gut mucosa has been neglected by biochemists.

One purpose of the present symposium, then, is to reemphasize the dynamic interaction among diet, function and form of the gut mucous membrane. This is no new observation—Schoenheimer presented evidence of the prompt interaction between ingested food and tissue chemistry (13, 14). The extent to which cellular morphology reflects alterations in nutritional and metabolic status, however, is only beginning to be sufficiently appreciated. This is evident in the relative paucity of morphologic controls used in biochemical investigations. Advances in morphologic methodology have speeded up the recognition of this truth. These include methods for labelling molecules with a variety of tags, both naturally occurring and synthetic. Only a few isolated examples can be given. The tagging of DNA with tritium-labelled thymidine (15) and the use of radio-phosphorus (16) have enabled anatomists to confirm elegantly the rapid turnover of the cells of the gut mucosa. Florey (17) demonstrated the active uptake by goblet cells of radio sulfur while adjacent nongoblet mucosal cells synthesized little sulphonated mucoprotein. On the other hand, Lipkin (18) showed the active uptake by nongoblet mucous cells (colon) of C^{14} labelled leucine. Histochemical methods are being developed to the stage of precise localization of cellular enzymes. Shnitka (19) reports on the applicability of some of these techniques to the gut mucosa. The electron microscope has brought exquisite confirmation of the functional significance of form. The light microscope alone, however, may be a valuable adjunct to nutritional and biochemical studies of the gut mucosa. Indeed, in our experience morphologic controls may be necessary in order to interpret correctly the results of a metabolic study.

If the surface epithelium of the rat gut is scraped off with a blade, a preparation is obtained consisting almost entirely of regular ribbons of columnar villus tip epithelium (fig. 2), leaving behind all of the tunica propria and the crypt epithelium (fig. 3). This preparation is readily studied in the Warburg flask. When animals are fed diets deficient in one or more nutrients (protein, calories, pantothenic acid or other vitamins, especially of the B group), the oxygen consumption of the tissue declines (20).

The effect may be magnified by the use of an appropriate analogue, such as aminopterin (21). If one subjects rats to an injection of aminopterin and kills them at increasing intervals, within 3 hours the normal mitoses of the crypt epithelium (fig. 4) are replaced by necrotic and damaged cells (fig. 5). The columnar epithelium of the villus, however, appears intact, despite the fact that the O_2 consumption of the villus tip epithelium falls within 1 hour, and in 3 hours drops to 25% of the control value (fig. 6). The value returns to normal within 48 hours. In this experiment, the antimitotic effect of aminopterin is promptly apparent in the crypts,

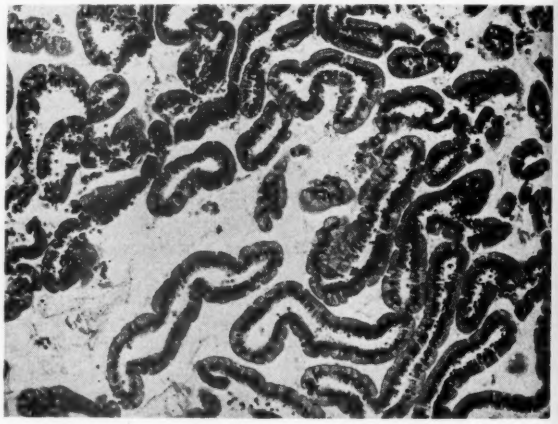


FIG. 2. Scrapings of villus tip columnar epithelium of rat small intestine. ×105.

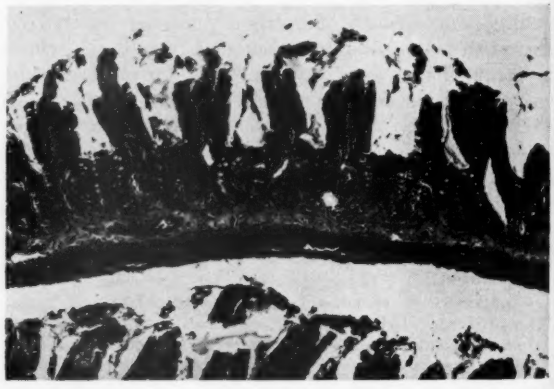


FIG. 3. Residual rat villus structure after scraping with blade, showing usual retention of intact underlying tunica propria. × 68.

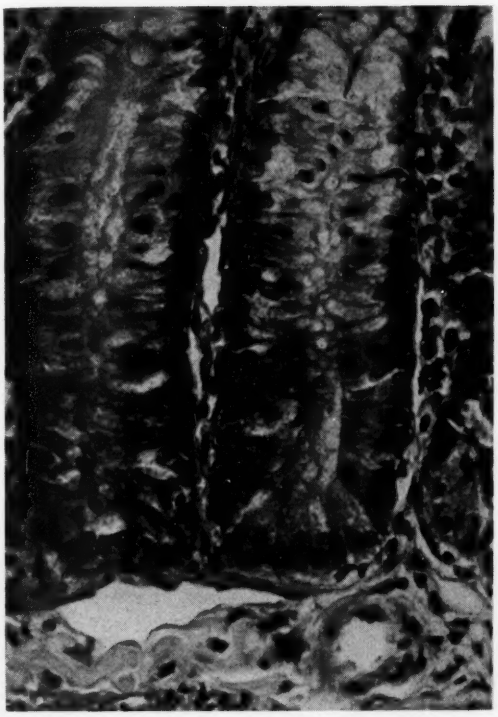


FIG. 4. Crypt epithelium with active mitotic regeneration. × 580.

but the morphology of the villus tip epithelium as viewed through the light microscope fails to show the biochemical lesions. If a smaller dose of aminopterin is given and repeated daily, one observes a slower fall in the O_2 consumption, reaching its depth in 48 hours, returning toward normal values by the fourth day, at which time a wide variation of individual values for respiration occurs (fig. 7). The significance of this variation becomes clearer when one examines the morphology of the tissue obtained at that time (fig. 8). The tissue is seen to be heavily infiltrated with inflammatory cells. A few abnormal mitotic figures are seen in the crypts and there is abundant infiltration with inflammatory cells. The normal ribbons of columnar epithelium are replaced by a flattened, abnormal tissue (fig. 9). It is now apparent that, in addition to the respiration of the abnormal columnar epithelium, our measurements include the respiration of the tunica propria with its infiltrate of actively respiring inflammatory cells. Repeated large doses of aminopterin destroy the mucosa (fig. 10), and the animal dies with bloody diarrhea.

The villi may appear swollen and blunted by the edema and inflammatory infiltrate of the underlying tunica propria, an appearance seen also in intestinal biopsies of patients with sprue. Actual reduction in numbers of intact villi occurs when an animal is given a nonfatal but severely damaging dose of aminopterin and allowed to recover. Adjacent villi devoid of mucosa appear to coalesce and become enclosed by new mucosa (fig. 11). Whether this mechanism is responsible for the similar appearance seen in biopsies of some patients with sprue remains to be studied. The studies of Sheehy *et al.* (22) suggest that the lesion, seen in malabsorptive states and in inflammations of the gut, is not specific.

Of interest was the fact that the pantothenic acid analogue, omega-methyl-pantothenate, alone, even in large doses, did not appear to damage the gut mucosa (23). When combined, however, with a dose of aminopterin, which, given alone, permitted survival for as long as 3 weeks, the animals died within 1 week (table 1). Similar effects were observed in respiration studies (table 2). Thus, 450 to 1500 micrograms of omega-methyl-pantothenate alone caused not more than a 21–27% fall in respiration of the small intestine. However, when the dose was combined with 120 micrograms of aminopterin, a marked (75%) fall in respiration was observed. The small intestine showed severe changes of the kind seen with aminopterin alone. Many of the colonic glands were found to be abnormally distended with mucus cells (figs. 12, 13). The lesser effect of the drugs on the mucosa of the colon compared with small intestine is probably due to the lower metabolic activity of the colon.

The effect of aminopterin on absorption of xylose was subsequently studied in rats (24). It was necessary to apply a dose of aminopterin too small to cause diarrhea or weight loss during the short time of the experiment. Such an experiment was achieved—indeed, the only evidence of drug effect consisted of infrequent abdominal foci in the crypts of the intestinal glands (fig. 14). Nevertheless, a significant reduction was observed in the absorption of xylose introduced directly into the small intestine (fig. 15).

The widespread use of D-xylose as a screening test for intestinal malabsorption is based, first, on the assumption that relatively little of the sugar is metabolized,

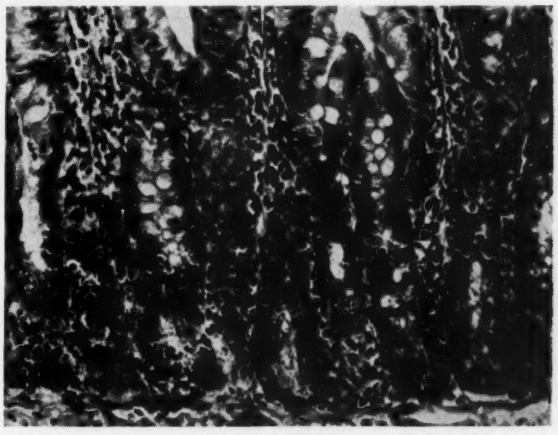


FIG. 5. Replacement of normal mitoses by degenerated cells, nuclear remnants and debris in rat sacrificed 6 and ¼ hr. after injection of 480 μg/kg aminopterin. (Lumen of crypts to top.) × 300.

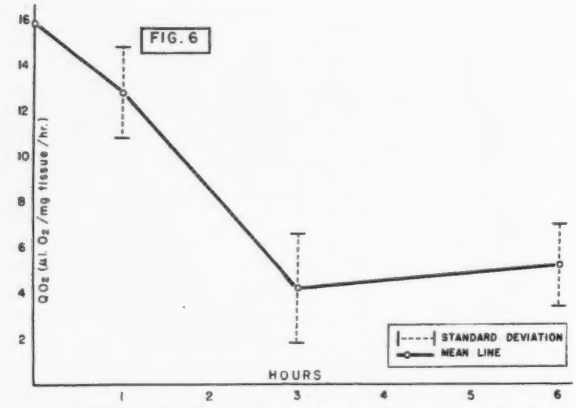


FIG. 6. Effect of aminopterin on duodenal mucosa respiration (dose—480 µg/kg body wt.). Each point represents mean of 3 animals.

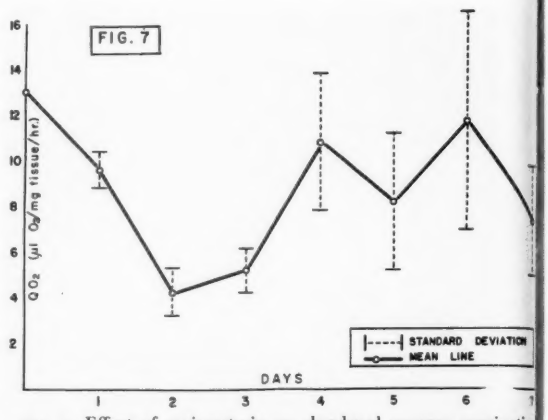


FIG. 7. Effect of aminopterin on duodenal mucosa respiratio (dose—120 µg/kg body wt/day). Each point represents mean 3 animals.

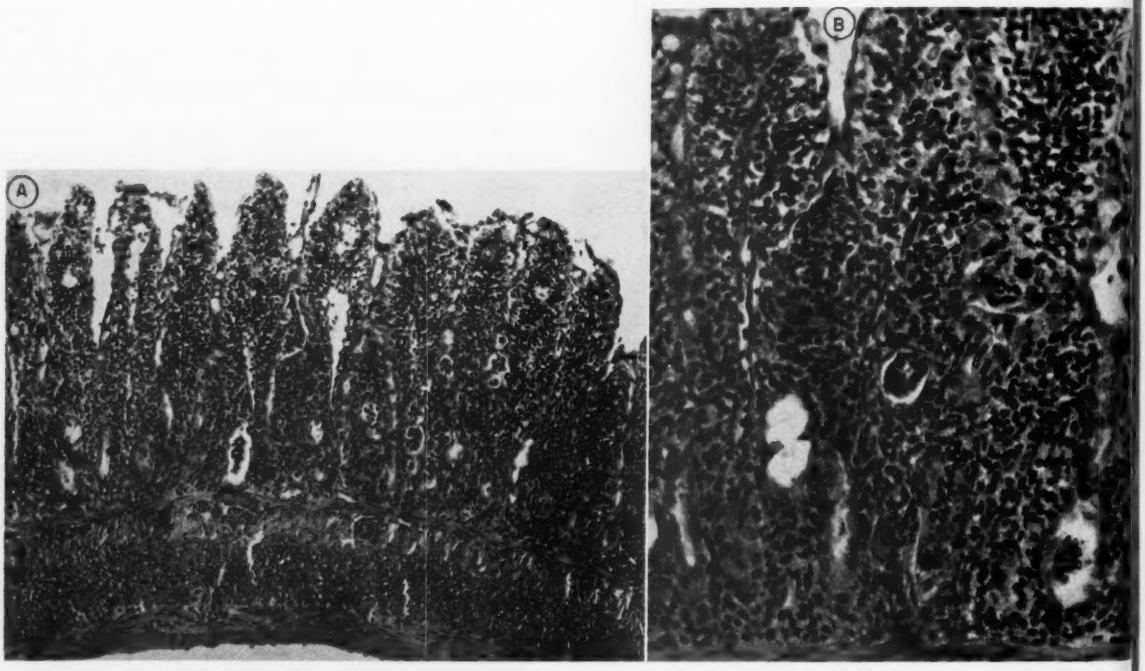


FIG. 8*A*. Duodenal villi heavily infiltrated with white blood cells. Crypts free of mitoses and plugged with white blood cells. Mu cosa flattened. Aminopterin, 480 µg/kg daily for 4 days. Animal moribund. × 170.

FIG. 8*B*. Magnification of fig. 8*A*. × 330.

and, secondly, that urinary excretion (in the absence of renal disease) reflects intestinal absorption. Several experiments were done to test the former assumption (25, 26). Neither a reduced (10%) protein diet nor ingested alcohol alone impaired absorption or excretion of xylose, but when both were given together, a decrease in the absorption and delayed excretion was observed (fig. 16). This confirmed our clinical findings of reduced xylose excretion in 18% of Skid Row alcoholic patients shortly after a drinking bout (27). Rats fed thyroxin excreted 60–70% more xylose within 5 hours than di control animals (fig. 17). Since direct measurements residual xylose in gut lumen revealed no significan difference in intestinal absorption, it was concluded that the increase in excretion reflected reduced metabo lism of xylose by body tissues. Thus, contrary to som previous opinion, the metabolism of xylose by bod tissues may be significant. Observations such as thes

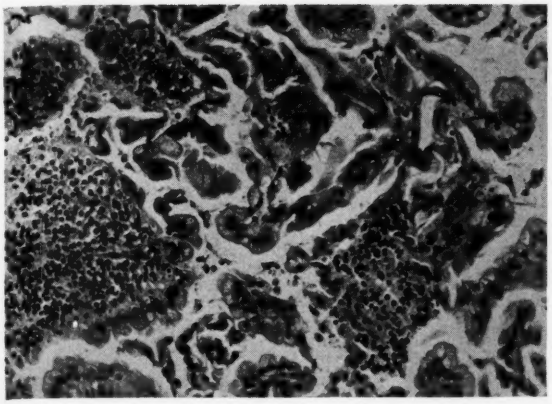


FIG. 9. Duodenal mucosal scraping showing effect of aminopterin. Distorted mucosal cells, dense tunica propria (connective tissue) infiltrated with round cells. Aminopterin 240 μg/kg daily; sacrificed fourth day. × 290.

FIG. 10. Intestinal mucosa entirely destroyed by aminopterin. Epithelium is replaced by hemorrhage and necrotic debris. Dark areas in upper portion of figure are pancreatic tissues. × 34.

supplemented by other clinical and laboratory findings, have caused us to question the reliability of the D-xylose test for diagnosis of intestinal malabsorption in some patients with nutritional or metabolic disorders (28).

The importance of species differences has been emphasized in several studies (29, 30) and has been reviewed recently (31). Large doses of aminopterin failed to produce either metabolic or morphologic changes in the guinea pig gut mucosa. The reason for this difference from rat mucosa was thought to be related to the differing folic and folinic acid contents of the gut mucosa of the two animals. Thus, while the rat had approximately 384 μg of folic acid and 161 of folinic acid per gram of small intestinal mucosa, the guinea pig had only 48 and 8 μμg of each, respectively. Exposure to aminopterin reduced rat gut folinic acid by almost 75%, but was without significant effect on guinea pig gut folinic acid. That mere tissue content of folic and folinic acid does not account entirely for the difference, however, was apparent from the fact that despite relatively large amounts of both folic and folinic acid in livers of rat and guinea pig, aminopterin had relatively little effect on these tissues. More likely, the effects were related to a more rapid turnover rate of the mucosal cells of the rat as compared with the guinea pig. The specific activities of radio-phosphoprotein obtained from mucosa of the duodenum, jejunum and ileum of rats were approximately 12, 11 and 5 times as great, respectively, as for the guinea pig tissues. Interestingly, we have observed more rapid rates of xylose absorption in the guinea pig than in rats (Vitale and Zamcheck, unpublished observations).

Measurements of succinoxidase activity of rat, guinea pig and human gastrointestinal mucosal homogenates also revealed important differences (32). Little or no activity was measurable in rat homogenates, whereas guinea pig and human mucosa provided relatively consistent and reproducible results. When histochemical studies indicated the presence of succinic oxidase activity, it was concluded that rat mucosa contained an inhibitor. The inhibition was subsequently shown to be

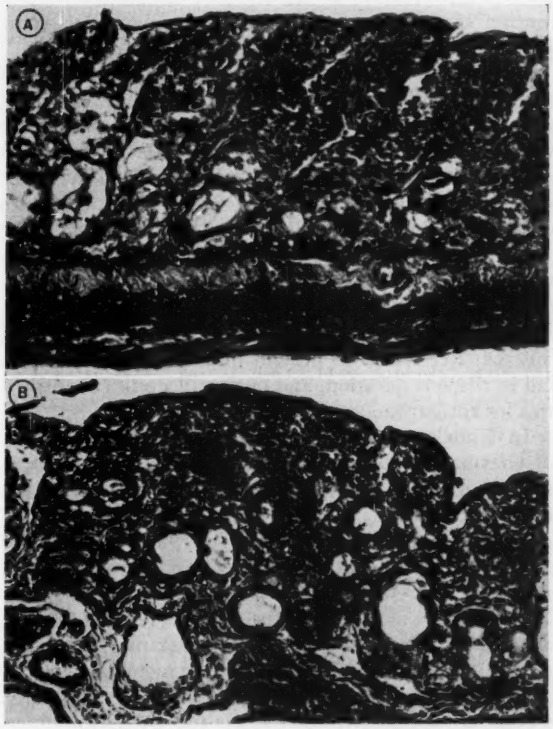


FIG. 11*A* and *B*. Two sections of intestinal epithelium of aminopterin treated rat showing evidence of recovery manifest by coalescence of adjacent tunicae propriae and regrowth of new epithelium. × 110.

TABLE 1. *Survival Time*

No. of Animals	Aminopterin, μg/kg	ω-Methyl Pantothenate, μg/kg	Life Span, days	Condition When Sacrificed
4	240	0	20–21*	terminal
4	0	750	20–21†	healthy
4	240	750	12–13	terminal
4	240	1500	6–7	terminal

* Not injected on days 13–16. † Not injected on days 12–13.

TABLE 2. *Respiration of Small Intestine In Vitro*

Aminopterin, μg/flask	ω-Methyl Pantothenate, μg/flask	No. of Flasks	QO_2 $\bar{x}$ ± S.D.	% Fall in Respiration
0	0	14	15.5±2.8	0
45	0	6	10.5±2.2	32
60	0	6	11.1±2.2	28
120	0	8	8.8±1.6	43
240	0	4	11.5±1.4	26
0	450	6	12.3±2.3	21
0	1500	10	11.3±2.3	27
45	450	5	12.9±2.3	17
120	1500	5	3.9±0.2	75

due to the presence of unsaturated fatty acids in the mucosa of the rat in relatively large amounts as compared to the guinea pig.

Differing effects in the rat and guinea pig of an atherogenic diet consisting of cholesterol and cholic acid were observed, both on serum cholesterol values as well as on the deposition of lipid in heart valves and aorta, and Vitale *et al.* (31, 33, 34) concluded that they may be due in part to differences in the absorption rates of steroids from the lumen of the gut.

Although there is abundant evidence relating alterations in diet to changes in enzyme activity, especially of liver tissue, there is relatively little information available on this matter with respect to intestinal mucosa. Reduction in protein intake commonly causes fall in tissue respiration, and our early studies confirmed this for rat gut mucosa as well.

In Knox's extensive survey (35), published in 1956, of enzymatic and metabolic adaptations in animals, a mere handful of the 752 references dealt with gut mucosa. Although, in general, tissue enzymes tend to respond to increased feeding of substrate by enhanced activity and to reduced intake by reduction of activity, this does not always occur. Relatively little specific information is available on the point as regards the gut, although it can hardly be doubted that the nature and amount of fed nutrients influences the metabolic activity of the gut mucosa directly, and of other tissues indirectly. Cohn and Joseph (36) have made the interesting observation that forced feeding of rats on a high carbohydrate diet—that is, compressing the feeding time—results in the increased use of the hexosemonophosphate oxidative shunt by the tissues studied (liver and epididymal fat). The authors conclude that the rate of ingestion of the diet plays a role in the regulation of traffic over specific enzymatic pathways when multiple alternate pathways are available. It would be of considerable interest to determine the effect of similar treatment on gut mucosal activity.

The dynamic interaction between the gut mucosa itself and the nutritional state of the host is apparent enough from animal experiments. Presumptive evidence of similar interaction in human experience is abundant if less precisely analyzable. Autopsy morphology of the gut mucosa is usually unsatisfactory because of the rapid autolysis of the tissue in the presence of digestive juices. The intestinal mucosa of patients with sprue shows changes which sometimes resemble those of experimental folic acid deficiency. Gardner (37) has observed cytologic changes of the squamous cells of the upper digestive tract epithelium consisting of enlargement of nuclei and cells and multi-nucleation, which have reversed on treatment with folic acid. Graham and

FIG. 12. Colon epithelium of rat treated with ω-methyl pantothenate and aminopterin showing altered epithelium of colonic glands which are distended with mucous-containing cells. × 220.

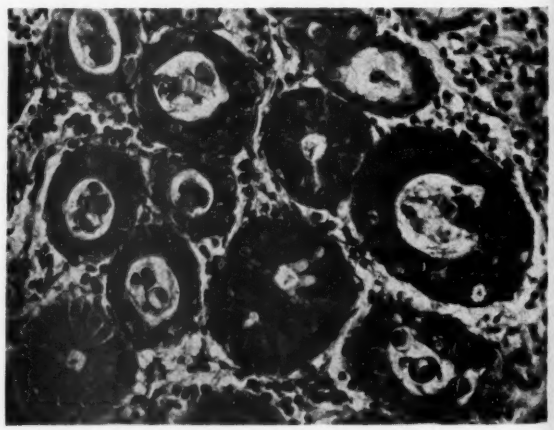


FIG. 13. Cross section of another area of fig. 12. × 330.

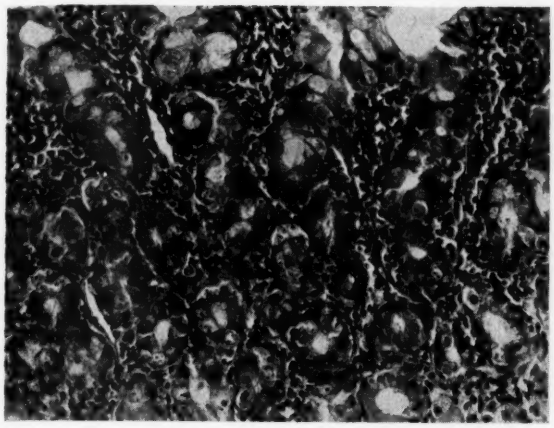


FIG. 14. Small intestine of aminopterin treated rat showing a focus of pathologic change in the crypt and villus mucosal cells; consisting of increase in size of nuclei containing prominent nucleoli and loss of uniformity. Occasional abnormal mitosis seen. × 225.

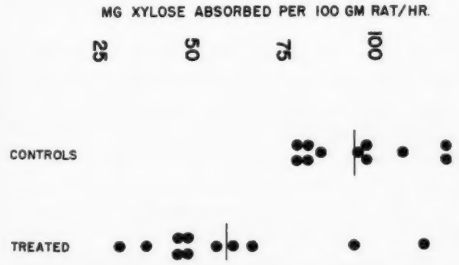


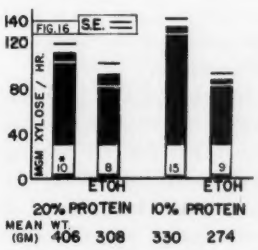
FIG. 15. Aminopterin vs. xylose absorption. Absorption of xylose in mg/100 gm rat/hour in normal and aminopterin-treated animals. Vertical lines represent means.

Rheault (38) have also shown similar changes in smears obtained in patients with pernicious anemia in relapse, which have reversed on therapy with liver extract or Vitamin B_{12}. Similar changes have also been described in the buccal mucosa (39).

Chronic alcoholic patients of the 'Skid Row' genre are highly susceptible to disorders of the gastrointestinal tract, including esophagitis, esophageal cancer, peptic ulceration and lacerations of the upper digestive tract, and gastritis, the lesions frequently manifesting themselves clinically as massive gastrointestinal hemorrhage (27, 40–45). To what extent these lesions are related to nutritional deficiencies and to what extent to alcoholic beverages is not entirely clear. The alcoholic patient commonly consumes a diet deficient in protein, vitamins and fat, but abundant in calories. We have reported some evidence of intestinal malabsorption in these patients, but the evidence is not unequivocal. Massive hemorrhage and diffuse ulcerations of the gut mucosa have been seen in other clinical conditions in which nutrition and metabolism have been impaired, such as congestive failure and uremia (46). It is entirely likely that nutritional deficiency or 'imbalance' contributes to these lesions, but the complexity of the clinical situation precludes analysis into specific nutrient deficiencies. Furthermore, the alcoholic patient frequently has multiple disorders of the digestive system, including fatty liver and pancreatitis as well as those of the gastrointestinal tract. Even when these disorders do not trouble the patient sufficiently to require medical attention, careful physical examination or appropriate laboratory testing brings out functional organ deficiency (27).

The availability of instruments for the ready biopsy of the gastrointestinal mucosa has already provided much information on the morphology of this tissue and promises more (47–49). The changes in the intestinal mucosa in sprue—'tropical' and 'nontropical' or idiopathic—and in coeliac disease have been described (50–54). The evidence to date suggests that the morphologic lesion in these several conditions is the same, or at least similar (55, 56). Rubin (57) considers the lesion an irreversible one, although there appears to be some evidence that the microvilli allegedly lost in sprue are repaired on the institution of a gluten-free diet (58). The series of studies by Dicke (59), and Weijers and Van de Kamer (60) clearly showed that dietary gluten does indeed cause malabsorption and that the elimination of gluten from the diet often reverses the functional deficiency. Rubin and co-workers (61) instilled wheat flour into the ileum of two sprue patients in remission and produced exacerbation of sprue symptoms, impaired absorption and the characteristic lesions of the ileal mucosa as demonstrated by biopsy studies. Although it was previously assumed that glutaminase activity was absent from the gut mucosa, it has been demonstrated recently in the mucosa of the rat (62). The relationship, if any, of this enzyme to intestinal malabsorption and the lesion produced by gluten has not been studied.

Atrophy of the small intestine is also described in kwashiorkor, but there has been little study of mucosal morphology or metabolism in this condition (63). The demonstration of atrophic changes in the intestinal mucosa of some patients following gastrectomy has helped resolve some of the uncertainty regarding the pathogenesis of malabsorption in the post-gastrectomy state (64).



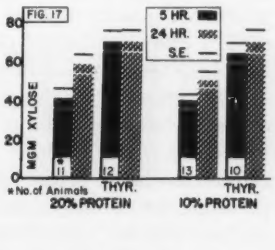


FIG. 16. Effect of alcohol on intestinal absorption of xylose. Numbers of animals given in white rectangles.

FIG. 17. Effect of thyroxine on urinary recovery of xylose. Number of animals given in white rectangles.

Diarrhea and gastrointestinal ulcerations have been associated with the use of potent analogues such as aminopterin, used in the treatment of malignancy (65, 66), but there is available relatively little good morphology and essentially no biochemical study of the human tissue.

We may expect useful information from biochemical studies performed on biopsies of human tissue obtained in this manner. Dawson and Isselbacher (67) have studied the tissue dynamics of fat absorption with the aid of this technique.

The nutritional literature is replete with references to the diverse effects of diets of varied composition on growth, tissues, enzymes and diseases of man and animals. Yet little is known about the influence of these diets on the metabolism of the gut mucosa itself, through which these effects are mediated, at least in part. One must conclude that it has been widely assumed in the past that the gut mucosa does little more than pass available nutrients from the outside world into the internal milieu. That this is clearly not so is evidenced by studies of the metabolism of this tissue. It is likely that some of the variable results of nutritional experiments may be ascribed to differing phenomena at the gut lumen-mucosal cell interface.

Integration of the myriad phenomena occurring at the gut-lumen-mucosal cell interface, however desirable, is impossible at the present state of knowledge. Large bodies of information are either unavailable or insufficiently understood even as isolated phenomena, let alone as participants in a highly complex biologic activity. Some of these large gaps deserve mention, since they are not treated in this symposium.

The bacterial flora of the gut actively influences the biology of this tissue by synthesizing vitamins and participating in metabolism in many ways. The feeding of antibiotic supplements to the diet is commonly practised in animal husbandry in order to promote maximal growth (68). Malabsorption has been produced in humans (69) and in rats (Small and Zamcheck, unpublished observations) by means of oral neomycin, presumably caused by suppression of bacterial flora. In rats, on the other hand, the absorption of cholesterol may be enhanced by means of this drug (70). Observations in our laboratory suggest that the role of some antibiotics may be exerted, in part at least, by reason of their effect on the pH of the gut contents (Broitman and Zamcheck, unpublished observations).

The influence of the gut lumen-mucosal interface on the metabolism of distant organs has been alluded to. Rutenberg *et al.* (71) have pointed out that the feeding of nonabsorbable broad spectrum antibiotics delays or prevents the development of cirrhosis in rats fed a choline deficient diet. They attributed this effect to the reduction or elimination of intestinal bacteria by the antibiotics. The beneficial effect of nonabsorbable antibiotics in the prevention of hepatic coma derives in part from suppression of enzymic hydrolysis of urea by bacteria in the release of ammonia. By limiting protein intake, production of ammonia and other nitrogenous metabolites is minimized (72–75). The extent to which the gut mucosal metabolism participates in ammonia production in this syndrome has not been studied (62).

Ahrens (76) is impressed with the importance of the role of intestinal bacteria in affecting serum lipid levels. Biliary cholesterol and bile acids undergo changes in the intestine due to bacterial action to yield mixtures of sterols (cholesterol, coprosterol and dihydrocholesterol) and ketonic bile acids. The type of intestinal flora is influenced by the diet and appears to determine the qualitative composition of the excretory products of cholesterol metabolism. Thus, the highly complex method for disposal of cholesterol depends, finally, on the action of the intestinal flora (77). Furthermore, reabsorption of undegraded bile acids unquestionably affects the rate at which cholesterol is converted to bile acids in the liver. Portman has described nutritional influences on the metabolism of the bile acids (78). Ingested alcohol and dietary magnesium also influence serum cholesterol and experimental atherogenesis (33, 34, 79).

Fine (80) has suggested that endotoxin produced by intestinal bacteria may be partly responsible for clinical as well as experimental shock. Intracardiac injection of lethal doses of endotoxin causes edema and congestion and microscopic hemorrhages of the rat small intestine within 20 to 80 minutes (81). Succinoxidase activity of small intestine homogenates is decreased within 30 seconds of endotoxin injection, the rapidity of action suggesting a direct effect of endotoxin on the gastrointestinal tract rather than one caused by tissue anoxia secondary to the vascular reaction. The fact that transmural migration of *S. marcescens* from the gut lumen did not occur despite morphologic damage and enzymatic impairment may be one indication of the effectiveness of the mucosal barrier under these experimental conditions. Little is known about the properties of the mucus which contribute to its "barrier" function. Dische (82) has described some of his observations in this important and difficult matter.

To study gut mucosal biology without considering these and other phenomena is to guarantee inadequate understanding. Other large areas of importance are the role of hormones, including histamine and serotonin, the mineral elements of the diet, such as calcium and magnesium which have intimate effects on the integrity of the gut mucous membrane, and interrelationship among dietary constituents (83). The system of integration par excellence is the antonomic nervous system. Its role in adjusting cellular metabolism has been little studied.

There is need for concerted effort in the design of experiments by means of which individuals trained in different disciplines may study the effects on several

variables of a given alteration. Not until such experiments are done shall we begin to understand the remarkably dynamic integration of biological systems. Some of the experiments reported here represent a very small step taken in this direction.

So much, then, by way of introduction. Time does not permit greater elaboration of the reason for interdisciplinary consideration of the biology of the gut mucous membrane or of the evidence of its validity. Suffice it to say to those concerned with the biology of this tissue and its relation to other tissues in normal and diseased man or animals, the necessity for comprehending its integrated activity becomes increasingly compelling.

During recent years at the Boston City Hospital, in the course of studies on both man and experimental animals, the necessity for a multidisciplinary attitude has become increasingly clear. The studies alluded to above were carried out by individuals with differing backgrounds. Some of these include: Dr. Joseph J. Vitale, Dr. Mark D. Hegsted, and Dr. Stanley Gershoff, nutritional biochemists of the Department of Nutrition, Harvard School of Public Health; Dr. Selwyn A. Broitman, microbiologist; Dr. Melvin D. Small, physiologist; and Drs. Leonard Gottlieb and Earl Hellerstein, pathologists. Several clinical residents and research fellows participated in the human studies, as indicated in the references.

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The first common pathway

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Before the building blocks and sources of fuel can gain access to the metabolic pools of the body, they must with but few exceptions follow the same common pathway: transport across the gastrointestinal epithelium. I would like to develop a picture of this barrier through which material must find a path.

The picture is not particularly novel but it is one that I believe to be essential for any rational framework. It is only an extension to the gastrointestinal tract of principles that have long been recognized in general physiology (1, 2). To gain access to the body, each and every material must cross the membranes of the gastrointestinal epithelium. These membranes are not radically different from those of other cells. They can be pictured as a 'lipoid-sieve' (3). The nature of the 'lipoid-sieve' is such that most of the area of the membrane has a 'lipoid' character permitting penetration of nonpolar lipid-soluble solutes which are capable of dissolving in this phase. This lipoid face is punctured by aqueous pores. But these pores are very small, perhaps having an effective radius of 3–4 Å, such that all water-soluble solutes having a molecular weight of 100–200 and greater are virtually excluded. No less important, these pores comprise only a very small fraction of the total surface area. A. K. Soloman (4) has estimated for the erythrocyte that they only constitute $1/10{,}000$ of the overall area. It might be added parenthetically, that for two reasons we believe that these are actual pores with a continuous aqueous phase. The movement of water across cell membranes, due to a difference in activity of water, is greater than that which is predicted from the isotopic diffusion of water. This inconsistency can be most simply explained by hydraulic flow. Secondly, a net movement of water can exert a significant drag upon a lipid-insoluble solute like thiourea (5). But having advanced arguments for the presence of such pores, I now wish to emphasize that these pores are unimportant in gastrointestinal absorption.

To picture the consequence of a barrier that is a 'lipoid-sieve,' it may be helpful to note the more important ways in which material moves across a membrane (6):

Passive diffusion—
- A. Through the lipoid phase
- B. Through aqueous pores

Stereospecific transport—
- A. Active transport uphill
- B. Facilitated diffusion downhill.

Material moves across the gastrointestinal epithelium primarily in one or another of two ways. It may move

by passive diffusion through the lipoid phase or it may be actively transported by a stereospecific mechanism. Passive diffusion through the aqueous pores is too radically restricted to be anything but inconstant. Stereospecific facilitated diffusion, though important for the entry of certain substrates such as glucose into many cells, has not been recognized as important in gastrointestinal absorption.

Together with Schanker, Shore and Brodie, I have sought to characterize the nature of the gastrointestinal barrier by examining the absorption and secretion of a particular group of foreign compounds: drugs. I shall speak of these compounds as foreign because they do not ordinarily participate in normal metabolism and as far as we know their specific pharmacologic properties are inconsequential in the studies I wish to review. They offered advantages They constitute a wide variety of chemical structures, available in high purity for which precise analytical techniques could be developed. In order to make an especial plea, I shall stress the classification of these compounds as weak electrolytes. This aspect of their property will be expressed by a pKa or negative logarithm of the acidic dissociation constant.

When a number of drugs are introduced into the rat stomach, the concentration being 1 mM and the volume 5 ml of 0.1 M HCl, a definite pattern of absorption can be recognized (7). If the cleansed stomach is isolated by tying off the cardiac and pyloric ends, almost all acidic drugs are absorbed substantially, up to 60% of that introduced into the stomach. However, the very strongly acidic compounds, those having a pKa less than 1, are not absorbed appreciably, table 1. Equally informative, it is only the very weakest basic compounds,

TABLE 1. *Absorption of Acidic Compounds by the Rat*

Acid	Absorption ml cleared/hr.			
	pKa	Stomach	Intestine	Colon
Phenol red	Strong	0	0	0
5-Sulfosalicylic	Strong	0	0	0
o-Nitrobenzoic	2.2		1	0
5-Nitrosalicylic	2.3	3.7	5	0.2
Salicylic	3.0	4.7	82	1.5
m-Nitrobenzoic	3.4		62	1.3
Acetylsalicylic	3.5	2.2	21	
Benzoic	4.2	4.0	70	2.5
Phenylbutazone	4.4		70	5.7
Thiopental	7.6	3.1	100	8.3
Secobarbital	7.9	1.8		6.1
Barbital	7.8	0.2	26	1.5
p-Hydroxypropiophenone	7.8	4.0	85	7.4
Phenol	9.9	2.6	82	5.4

These compounds, 1 or 0.5 mM/l, were dissolved in solutions of different pH; stomach 1, small intestine 6.6 and colon 6.8. Clearance was computed from the equation

$$\text{Clearance} = \frac{\text{Volume}}{\text{Time}} \ln \left(\frac{\text{Initial Concentration}}{\text{Final Concentration}} \right)$$

(Compiled from Schanker *et al. J. Pharmacol. Exptl. Therap.* 120: 528, 1957; 123: 81, 1958 and Schanker ibid. 126: 283, 1959.)

TABLE 2. *Absorption of Basic Compounds by the Rat*

Base	Absorption ml cleared/hr.			
	pKa	Stomach	Intestine	Colon
Acetanilide	0.3	2.2	51	5.4
p-Nitroaniline	1.0		85	8.3
Antipyrine	1.4	0.8	32	2.7
m-Nitroaniline	2.5	0.9	89	7.8
Aniline	4.6	0.3	70	7.0
Aminopyrine	5.0	0.1	28	2.8
p-Toluidine	5.3	0	74	8.6
Quinine	8.4	0	8	2.7
Levorphan	9.2	0		1.7
Ephedrine	9.6	0.1	3	1.1
Tolazoline	10.3	0	1	0.5
Mecamylamine	11.2	0	3	0.4
Darstine	Strong	0	0	
Tetraethylammonium	Strong	0	0	0
Procain amid ethobromide	Strong	0	0	0

See Table 1.

TABLE 3. *A Comparison of Human and Rat Gastric Absorption*

Compound	Gastric Absorption (ml of gastric content cleared/hr./m²)	
	Human	Rat
Salicylic acid	220	170
Deuterium oxide	170	200
Thiopental	150	110
Aspirin	110	90
Ethanol	100	50
Secobarbital	70	50
Antipyrine	50	30
Ephedrine	0	3
Aminopyrine	0	0
Quinine	0	0

These clearances are expressed per square meter of body surface area assuming that $m^2 = 0.1$ (kg b.w.)$^{2/3}$. (Compiled from Hogben *et al. J. Pharmacol. Exptl. Therap.* 120: 540, 1957.)

those with a pKa less than 3, which are absorbed detectably, table 2. This pattern of absorption suggests a tentative interpretation. Those acidic and basic compounds which are absorbed are those which are substantially unionized or present in their water-soluble or lipid-insoluble form. This leads to a simple explanation. Gastric absorption occurs by simple passive diffusion of the unionized moiety of a weak electrolyte across a gastric mucosa which is preferentially permeable to this form and impermeable to the ionized form. If absorption is expressed as a clearance relative to body surface area (8), we find a surprising similarity between absorption from the rat and human stomach, table 3. A further qualitative confirmation of the explanation for the absorption of foreign compounds can be obtained by contrasting gastric absorption of several drugs from solutions of different pH; 1 and 8 (7). Changing the pH of the solution introduced into the stomach to 8 (isotonic bicarbonate) increases the ionization of acidic and decreases that of basic compounds. Consequently, and as expected, the absorption of acidic compounds

decreases and the absorption of basic compounds increases.

This interpretation of the gastric mucosa relies on an essentially qualitative interpretation of data. The reason for this will be commented upon later. A much more exacting scrutiny of the hypothesis that the mucosa is selectively permeable to unionized drug can be obtained by study of the gastric secretion of foreign compounds (9). If the gastric mucosa is considered to be equivalent to a lipid barrier separating two aqueous phases, the steady-state distribution of a weak electrolyte will be that indicated by figure 1 in (9). In the steady state, the unionized form which is able to diffuse with ease across the barrier will have the same concentration in the two aqueous phases. The pH of each phase, 1 (gastric juice) and 7 (plasma), will determine the concentration of the ionized moiety relative to the unionized. For this model the ratio, R, of total drug (ionized plus unionized) in gastric juice relative to that in plasma is given by the following two equations (9):

$$\text{for acids } R = \frac{1 + 10^{(1-pKa)}}{1 + 10^{(7-pKa)}},$$

$$\text{for bases } R = \frac{1 + 10^{(pKa-1)}}{1 + 10^{(pKa-7)}},$$

If a drug is given by continuous infusion to a dog with a Heidenhain pouch, it is possible to determine experimentally the value of R, the concentration of drug in pure gastric juice relative to that in systemic plasma (corrected for plasma binding). A comparison of the values actually found with those which are predicted by the above equations is given in table 4. The model predicts that stronger acids would not be detectably present in gastric juice. In the case of the weaker acids and bases there is excellent agreement between theory and experiment. It is only in the case of the stronger bases that there appears to be a discrepancy between prediction and observation. But before proceeding to a consideration of this apparent discrepancy it is appropriate to comment on the secretion of the weaker acids into gastric juice. The model that accounts for gastric secretion of foreign compounds which we have just examined was previously considered and rejected by Ingraham and Visscher (10, 11) in the course of their study of the gastric secretion of dyes. They rejected this model because they failed to detect the presence of weak acidic dyes in gastric juice when the dyes were given parenterally. This failure can be attributed to plasma protein binding and to the limited sensitivity of their methods. They drew particular attention to the failure of phenol red (phenolsulfonphthalein) to be secreted into gastric juice. In this specific instance the dye is actually a very strong acid (12), and it should not be secreted into gastric juice even though it has a color change corresponding to its second dissociation constant; pKa 7.9.

The apparent discrepancy represented by the observed ratio of the stronger bases in gastric juice relative to systemic plasma can be explained simply. The comparison undertaken is that with systemic plasma which was withdrawn from the jugular vein. Thus the concentration of base in gastric juice is compared to its concentration in plasma coming to rather than leaving the stomach. The model, however, predicts the ratio that should obtain after the mucosal blood has exchanged with gastric juice and after almost all of the stronger bases had passed into gastric juice. Thus, the amount appearing in gastric juice would be blood flow limited and the concentration ratio, gastric juice:systemic plasma, would become constant if the rate of formation of gastric juice were maximal as was the case in these experiments due to the use of histamine. In

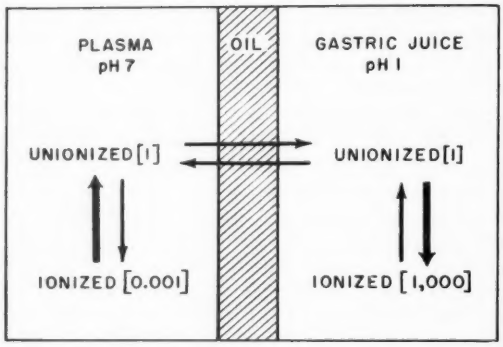

Fig. 1. Hypothetical plasma/gastric juice partition of base (pKa 4) (From Shore *et al.*, 9).

TABLE 4. *Comparison Between Observed Secretion of Weak Electrolytes into Canine Gastric Juice and that Predicted if Gastric Juice and Plasma Are Separated by a Lipid Barrier*

	pKa	Concentration Ratio (R). Gastric Juice: Plasma	
		Observed	Predicted
Stronger acids			
Salicylic	3.0	0	10^{-4}
Probenecid	3.4	0	10^{-4}
Phenylbutazone	4.4	0	10^{-3}
Weaker acids			
p-Hydroxypropiophenone	7.8	0.5	0.6
Thiopental	7.6	0.5	0.6
Barbital	7.8	0.6	0.6
Weaker bases			
Acetanalide	0.3	1.0	1.0
Theophylline	0.7	1.3	1.5
Antipyrine	1.4	4.2	4.2
Stronger bases			
Aniline	4.6	40	10^4
Aminopyrine	5.0	42	10^4
Quinine	8.4	38	10^6
Levorphan	9.2	40	10^6

Secretion is expressed in terms of the ratio, R, of concentration in gastric juice to that in systemic plasma. Plasma concentrations have been corrected for protein binding except for the stronger bases. (Compiled from Shore *et al. J. Pharmacol. Exptl. Therap.* 119: 361, 1957.)

fact, it would be hard to explain the surprising constancy of the gastric juice: systemic plasma concentration ratio of the several stronger bases on any other basis. The correctness of this explanation can be examined by measuring the gastric arteriovenous difference of one of these bases. The concentration of blood aniline is reduced to one-third in the gastroepiploic vein as it leaves the secreting stomach. While this is less than complete clearance, it must be recognized that it is only a portion of the blood going to the stomach that actually comes into contact with the gastric mucosa, for a fraction must supply the musculature and another fraction is presumably diverted through submucosal arteriovenous anastomoses.

Before leaving the matter of the secretion of stronger bases, I would like to comment on their use to clarify the absorption of foreign compounds from the stomach. When a diversity of compounds was studied, it was noted that there appeared to be an upper limit to the rate at which compounds were absorbed from the rat stomach: a clearance of 5 ml/hr. This upper limit was approached by a number of compounds of different chemical structures, lipid solubilities and dissociation constants. There are three obvious limiting steps: diffusion in the volume of fluid contained within the stomach, transition across the gastric epithelium and finally the limit imposed by gastric mucosal blood flow. The clearance of a stronger base offers the chance to evaluate the last of these three possibilities because the secretion of a stronger base into gastric juice is clearly a minimal estimate of gastric mucosal blood flow.

Anesthetized rats were prepared to permit the introduction and removal of a solution containing deuterium oxide and salicylic acid. These solutions were analyzed for the appearance of aniline. These changes have been expressed as clearances in table 5. The clearance of aniline into the stomach exceeds the rate of removal of deuterium oxide and salicylic acid by a factor greater than 10. Clearly we can dismiss blood flow as limiting absorption in the anesthetized rat. Because the upper limit of absorption is approached by many diverse compounds we are reluctant to presume that the gastric mucosa constitutes the limit to very rapid absorption. By default, we attribute the restriction to the diffusion within the volume of solution contained by the stomach. But it must be noted that these observations were conducted under conditions where normal motility which would lead to more adequate mixing had been suppressed by anesthesia.

It might be stated, by way of emphasis, that it is the gastric secretion of drugs that has offered us the opportunity to subject the 'lipoid-sieve' description of the gastrointestinal tract to closest scrutiny. It is not that subsequent observations are at variance with this description but that, in order to achieve quantitative conformity, it is necessary to introduce the further speculation that a 'microclimate' exists at the epithelial surface which is less accessible to direct verification.

We will now consider the absorption of foreign compounds from the small intestine (13). When an almost neutral saline solution is perfused *in situ* through the entire small intestine of the rat, most drugs are rapidly absorbed. The maximal rate of absorption from the small intestine of the anesthetized rat approaches a clearance of 100 ml/hr., tables 1 and 2. This rate, which is 20-fold greater than that reached in the stomach, may possibly be increased even further in the unanesthetized rat by normal motility. The same general rule obtains: unionized compounds are absorbed rapidly and ionized are not. In contrast to the stomach, because of the higher ambient pH bathing the intestinal mucosa, there is a shift in the critical pKa separating rapidly absorbed compounds from those that are less rapidly absorbed. The stronger acidic drugs having a pKa less than 3 and the stronger bases having a pKa greater than 7 are not rapidly absorbed. And when the absorption of the less rapidly absorbed drugs is examined by continuously recirculating the solution through the intestine, the same conclusion is reached. The dissociation constant of a weak electrolyte is a prime determinant of its rate of absorption.

When the pH of the solution perfused through the intestine is changed, the absorption of drugs is altered in a predictable fashion (14). Decreasing the pH to 4, which in turn decreases the ionization of most acidic drugs and increases the ionization of most basic drugs, increases and decreases the rates of absorption of the two groups, respectively. Conversely, increasing the pH to 8 decreases the absorption of acidic drugs and increases that of basic drugs. In support of the view that absorption occurs by passive diffusion, it may be mentioned that the fraction absorbed is independent of concentration. No evidence of saturation kinetics was obtained for salicylic acid absorption over a 20-fold range of concentration and for aniline over a 200-fold range. Qualitatively the intestinal mucosa is similar to the gastric mucosa. It is selectively permeable to unionized particles and impermeable to ionized or water-soluble solutes.

So far, we have interpreted the absorption of these foreign compounds as occurring by passive diffusion

TABLE 5. *Relative Clearance of Aniline, Deuterium Oxide and Salicylic Acid by the Rat Stomach*

Clearance, ml/hr.	
Aniline	74±20
Deuterium oxide	5·5±2.2
Salicylic acid	4·9±1.8
Body weight, gm	253±24
Volume of solution recovered from stomach, ml.	5·5±1.9
Time interval, min.	20–50
Change of inulin concentration in the stomach	0.94–0.96

Clearance of aniline into the stomach is a minimal estimate of gastric mucosal blood flow. Clearance of deuterium oxide and salicylic acid is a measure of their removal from the stomach by absorption. Most of the values are given as the mean ± the range. (Compiled from Schanker *et al. J. Pharmacol. Exptl. Therap.* 120: 528, 1957.)

of the unionized moiety. We have also suggested that if the unionized moiety is sufficiently lipid-soluble and if the ambient pH is favorable, they may be so rapidly absorbed that the permeability of the gastrointestinal epithelium is no longer the limiting factor. These remarks are sufficient to describe the broad principles governing the absorption of foreign compounds.

But if one wishes to make a more detailed inquiry into the movement of weak electrolytes across the gastrointestinal epithelium, it is necessary to take cognizance of the alterations of pH which occur in association with active transport of strong electrolytes. This is obvious in the case of the stomach where secretion leads to elaboration of a solution pH 1. There are also smaller but significant pH changes associated with salt absorption from the small intestine and colon. I would like to advance the proposition that these alterations of pH can be significant when we introduce into the lumen a solution whose pH does not coincide with that at the site of absorption.

As you have noted, the distribution of weak electrolytes between gastric juice and plasma is in excellent accord with the thesis that these two solutions are separated by a barrier that is selectively permeable to the unionized form. However, if the flux of a weak electrolyte across the isolated gastric epithelium is studied, we do not find such excellent agreement (15). In fact, even when the saline solution bathing the mucosal surface is more alkaline than that bathing the serosal surface, there is net movement of a weak acid, such as lactic acid, from mucosa to serosa. This anomalous movement ceases and is reversed when spontaneous secretion is blocked. One explanation is that the pH at the site of absorption during secretion remains largely independent of the bulk solution bathing the mucosa. Because it is partially independent we have spoken of this as a 'micro-climate.'

We can undertake to determine whether there is a comparable situation obtaining in the small intestine; an apparent pH at the site of absorption which is different from the pH of the bulk solution within the lumen. The equations given on page 866 describe the ideal steady state distribution of a weak electrolyte between two solutions of known pH. But if the distribution has been determined, the equations can be solved to yield the apparent pH at the site of absorption. The observed steady state distribution between the intestinal lumen and plasma of a drug, such as aminopyrine, leads to an estimate of the pH at the site of absorption which is 5.3 when the lumen is perfused with a poorly buffered saline having a pH of 6.6 (14). Perfusion of the intestine with highly buffered solutions of different pH does influence the steady state distribution of salicylic acid and quinine, but discrepancies remain between the observed and expected steady state ratios, table 6. The persistence of the discrepancies suggests that the somewhat acidic 'micro-climate' is partially independent of the pH of the solution in the lumen. There are two immediate reasons for developing this concept of an intestinal mucosal 'micro-climate.' Its presence precludes the simple type of quantitative analysis undertaken in the study of the gastric secretion of drugs. Secondly, it does serve to rationalize the critical pKa dividing the rapidly absorbed from the less rapidly absorbed drug. When absorption is occurring from poorly buffered saline, pH 6.6, the sentinel drugs are salicylic acid, pKa 3.0, and quinine, pKa 8.4, tables 1 and 2. These values are more nearly equidistant from the pH of the 'micro-climate', 5.3, in contrast to the pH of either the bulk solution within the lumen or that of plasma. While these observations justify consideration of the 'micro-climate,' I would not like to depart from this topic without suggesting that the concept may also be relevant to problems in gastrointestinal absorption and secretion other than that of weak electrolyte movement.

If absorption occurs by passive diffusion through the lipoid face of the gastrointestinal epithelium, one would naturally expect a correlation between rates of absorption and the lipid solubility of the unionized moiety. Some degree of correlation does exist (14). However, there is a serious obstacle to this type of study. If one compares the solubilities of a very heterogeneous group of compounds in different lipids, one finds that the ranking of the partition coefficients is not the same for different solvents such as chloroform and heptane. However, if one confines the analysis to a group of closely related compounds one can reasonably expect a

TABLE 6. *Effect of Intralumen pH Upon the Steady State Distribution Across the Small Intestinal Epithelium*

Lumen pH	Concentration Ratio Intestinal Lumen : Plasma	
	Observed	Expected
	Salicylic Acid	
4.3	0.13	10^{-4}
7.1	0.23	0.5
7.8	0.44	2.5
	Quinine	
4.3	36	10^{3}
7.1	23	1.9
7.8	3.5	0.4

The expected distribution is that calculated for distribution between the two solutions of known pH: plasma and intralumenal. (Compiled from Hogben *et al. J. Pharmacol. Exptl. Therap.* 125: 275, 1959.)

TABLE 7. *Correlation Between Rate of Absorption of Oxybarbiturates from the Rat Colon and Chloroform to Water Partition of the Unionized Moiety*

Oxybarbiturate	pKa	% Absorbed	Chloroform / 0.1 M HCl
Barbital (veronal)	7.9	12	0.7
Aprobarbital (alurate)	7.9	17	4.9
Phenobarbital (luminal)	7.4	20	4.3
Allylbarbituric acid (sandoptal)	7.7	23	10.5
Butethal (neonal)	7.9	24	11.7
Cyclobarbital (phanodorn)	7.5	24	13.9
Pentobarbital (nembutal)	8.1	30	28.0
Secobarbital (seconal)	8.1	42	50.7
Hexethal (ortal)	7.8	44	>100.0

(Compiled from Schanker, L. S. *J. Pharmacol. Exptl. Therap.* 128: 115, 1960.)

better correlation. Schanker (16) has found that the absorption from the colon of oxybarbiturates closely parallels the partition of the undissociated moiety between chloroform and water, table 7. This same study also demonstrates that absorption from the colon is qualitatively similar to that from the stomach and small intestine. The rates or clearances are more comparable to those encountered in the stomach, tables 1 and 2. The pH of the colonic mucosal 'micro-climate' was estimated to be 6.5 tending to restrain absorption of some of the stronger acids and favor that of some of the stronger bases. Thus the permeability of the large intestinal mucosa is such that the colon is not ordinarily an important site for the absorption of foreign compounds. Except perhaps for some moderately stronger bases, if absorption has not occurred higher in the gastrointestinal tract, circumstances will not be especially favorable for further absorption from the colon.

Having delineated the restriction imposed upon absorption by the gastrointestinal epithelium, it should be noted that this restriction is only part of the problem of absorption. In the work reviewed, the study was greatly simplified by restricting attention to absorption from true solution. But it is only after a compound has become molecularly dispersed that the general principles covered above will govern absorption. Clearly a compound may be inherently insoluble in the lumen, its physical form may not favor solution or the compound will not dissolve because of the pH of the gastrointestinal contents. The motility of the gastrointestinal tract influences absorption in several ways. There is reason to suppose that absorption can be delayed by diffusion through the fluid contained within the lumen. Mixing will diminish this restraint. The volume of the fluid and its rate of propulsion through the different segments of the alimentary tract cannot be ignored.

But even though we have to consider factors other than permeability, we may still draw some inferences from the nature of the first common pathway. Evidence indicates that the epithelial membrane is essentially impermeable to passive diffusion of water-soluble solutes having a molecular weight greater than 100. Since very few materials of interest in metabolism are sufficiently small and many are water soluble, other mechanisms must be sought to account for their penetration. The stereospecific transport systems for monosaccharides and aminoacids explain the absorption of much of our food. To these may be added a mechanism for the active transport of pyrimidines (17) and the specific process which is less clearly defined for the absorption of B-12. But what of the absorption of many materials such as the other water-soluble vitamins? Even though their absorption has not been clarified, it is reasonable to predict that stereospecific transport systems will be discovered for these vitamins.

The absorption of trace amounts of materials that cannot easily permeate the gastrointestinal epithelium should not occasion surprise. We have only a qualitative picture of the average pore diameter. But in the light of what we now know about the permeability of the intestinal epithelium, the appreciable absorption of intact protein is surprising and unexplained (18). While there has been much mention of the possibility of pinocytosis with little obvious applicability, this process may have to be considered in this context.

The absorption of lipids is enigmatic for other reasons. I am quite prepared to state that the gastrointestinal epithelium does not present a barrier to the diffusion of molecularly dispersed lipid. If absorption is incomplete or requires particular conditions, we must conclude that the lipids are too poorly soluble in the gut contents or that in the case of naturally circulating lipids the extracellular fluids are saturated. This negative observation does not impel me to accept the proposition that lipids move across the intestinal epithelium in aggregate form. The static pictures of electron microscopy cannot weigh heavily against the failure to find evidence of particulate absorption (19). If we wish to define the molecular events responsible for lipid absorption we must gain an explicit knowledge of the physical-chemistry of the solutions bathing the intestinal epithelium. But until such information is available and even though we must admit of the possibility of migration in an aggregate form we should emphatically challenge the suggestion that there might be active transport systems to effect the transfer of solutes through a membrane which is inherently extremely permeable to the same solutes.

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Intestinal transport of sugars and amino acids[1]

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A VARIETY OF EXPERIMENTAL APPROACHES are available for the investigation of intestinal absorption, each having its own special usefulness. The balance method, for example, is the only feasible approach for the study of absorption of many compounds in normal human subjects. Other *in vivo* methods may be employed for studies of unanesthetized animals. These include the use of the Thiry-Vella loop, the Cori technique (1) and the portal vein cannula method of London (2). In general, anesthetic agents do not seem to affect absorption (3–5), and consequently much important information has been obtained with anesthetized animals.

It was obvious, however, to the earliest investigators that *in vitro* methods would allow a wider range of experimental conditions than those obtained *in vivo*. As early as 1892 Reid (6) studied salt absorption in isolated rabbit intestine. Subsequently, a number of other workers have reported studies on isolated tissues (7, 8), but inadequate oxygenation limited the usefulness of such preparations. In 1949 Fisher and Parsons (9) overcame this difficulty by circulating oxygenated Krebs-Ringer solution separately on the two sides of isolated rat small intestine. This work stimulated a number of investigators to explore other *in vitro* methods (10–13).

One such *in vitro* preparation was the everted sac technique developed in collaboration with Dr. Wiseman (13). As many of the results to be presented were obtained with this method, a brief description of the technique is appropriate. The essential feature of this method was the eversion of the intestine so that the epithelial cells, which have a high rate of respiration, were directly exposed to the oxygenated incubation medium. The eversion was accomplished by means of a long stainless steel probe as previously described (13). A 2–4 cm segment of tissue tied at one end was filled with about 1 ml of solution from a syringe attached to a blunt needle. The second end was then tied. A sac of hamster jejunum prepared in this manner is shown in figure 1. The sac was incubated in a 50 ml Erlenmeyer flask containing usually 5 ml of solution and gas phase of 5% CO_2 and 95% O_2. Such a preparation was found to maintain a number of physiological functions for 1 hr. at 37° with no detectable morphological change in the epithelial cells. Sacs of everted intestine of the rat, hamster or frog transported a number of substances which the intestine is known to absorb in the living animal, including sugars (13), amino acids (14), water and inorganic ions (15–18), lipids (19, 20), nucleic acid derivatives (21, 22), and vitamins (23, 24).

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SUGAR ABSORPTION

An experiment illustrating the application of this method to the problem of sugar absorption is presented in figure 2. When glucose was placed at equal concentrations on the two sides of the intestinal wall, the concentration fell on the mucosal side and rose on the opposite side during the incubation period. As little fluid movement occurred in this experiment, the concentration changes represented a net transport of glucose across the full thickness of the intestinal wall against a concentration gradient of 10 fold. Gradients of 100 fold were often observed, and the maximum gradient which the intestine could develop has never been determined as the glucose concentration on the mucosal side frequently decreased to levels indistinguishable from zero. Figure 2 also shows that aerobic metabolism was necessary to provide the energy required for transport against a gradient.

Early studies of Héden (25), Negano (26) and especially Cori (1) demonstrated that glucose and galactose were absorbed from the lumen of the intestine more rapidly than pentoses and other hexoses. Csáky (27) further showed that the rate of absorption of 3-o-methylglucose was similar to that of glucose while the corresponding 2-o-methyl, 5-o-methyl and 6-o-methyl derivatives were absorbed at much slower rates. Another important observation was that glucose (28) and 3-o-methylglucose (29) could be absorbed against concentration gradients *in vivo*. The development of the everted sac method permitted the study of intestinal transport of a wide variety of synthetic compounds, many of which were available in only very small quantities. More than 50 sugars and sugar derivatives have been examined with sacs of hamster intestine (30–34, and unpublished observation by C. R. Jorgensen). Figure 3 shows the transport of several compounds which may be considered modifications of the glucose molecule at carbon atom 1. One interesting compound is 1-deoxy-

FIG. 1. Tied sac of everted jejunum of the hamster.

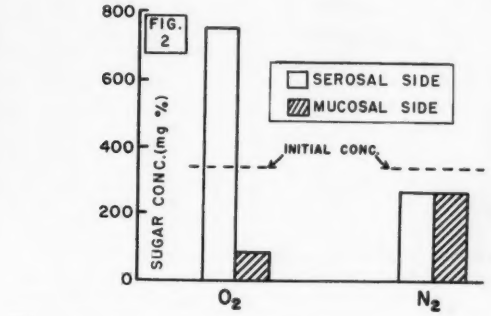
glucose[4] which was found by Crane and Krane (31) to be well transported. Two other compounds with modifications at carbon 1, α or β-methylglucoside, were also transported. It was concluded from such data that the hydroxyl group at carbon 1 was not essential for transport.

In contrast, the hydroxyl at carbon 2 appeared to be essential. The replacement of the hydroxyl group by hydrogen, as in 2-deoxyglucose, (fig. 4) produced an inactive compound. Other compounds with modifications at carbon 2 which were not transported include 2-deoxygalactose, 2-O-methylglucose, mannose, glucosamine and acetylglucosamine. The hydrogen on carbon 2, however, could be replaced without loss of activity as suggested by the active transport of 2-C-hydroxymethylglucose (33).

Additional experiments with other compounds indicated that the hydroxyl groups at carbons 3, 4 and 6 were not essential. Furthermore, none of the pentoses tested were transported. The important features appear to be a D-pyranose ring structure with a hydroxyl group of the glucose configuration at carbon 2. It should be noted that this generalization was based on alterations of single substituents on the glucose molecule. Changes of configuration at more than one carbon atom might give rise to inactive compounds, even though single modifications were tolerated (e.g. D-gulose which differs from glucose in the configuration of the hydroxyl groups at carbons 3 and 4 was not transported). Furthermore, compounds with sufficiently large substituent groups were not transported (34), probably because of steric effects or effects on the conformation of the sugar (e.g. 3-O-ethyl, 3-O-propyl and 3-O-butylglucose).

Before any general conclusions relative to the specificity of the transport system can be derived, it is important to determine whether all transported sugars share a common pathway. Other investigations have demonstrated competitive inhibition between glucose and galactose (35, 36) and between 3-O-methylglucose and glucose (37). Crane and Mandelstam (personal communication) found that 1-deoxyglucose inhibited glucose accumulation by segments of isolated intestine, although Keston and Tyree (38) failed to observe inhibition with this sugar combination with the sac method. Additional combinations of sugars have recently been

[4] More properly designated as 1,5-anhydro-D-glucitol.



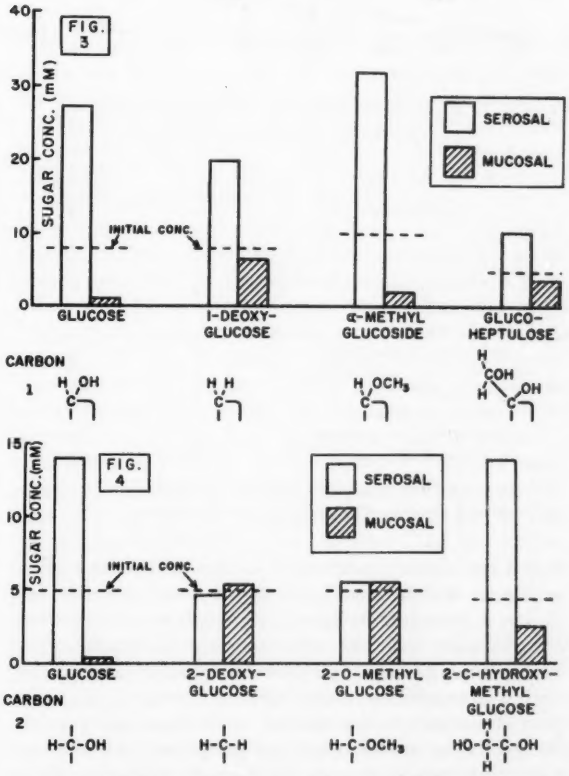


FIG. 2. Glucose transport by everted sacs of jejunum. One sac was incubated in an atmosphere of 5% CO_2 in O_2, the other with 5% CO_2 in N_2.

FIG. 3. Effect of modifications of the substituents of carbon 1 of the glucose molecule on active transport. These figures were constructed from published data for 1-deoxyglucose (31), α-methylglucoside (34) and glucoheptulose (32).

FIG. 4. Effect of modifications of the substituents of carbon 2 of the glucose molecule on active transport. These figures were constructed from published data for 2-deoxyglucose (32), 2-O-methylglucose (34) and 2-C-hydroxymethylglucose (33).

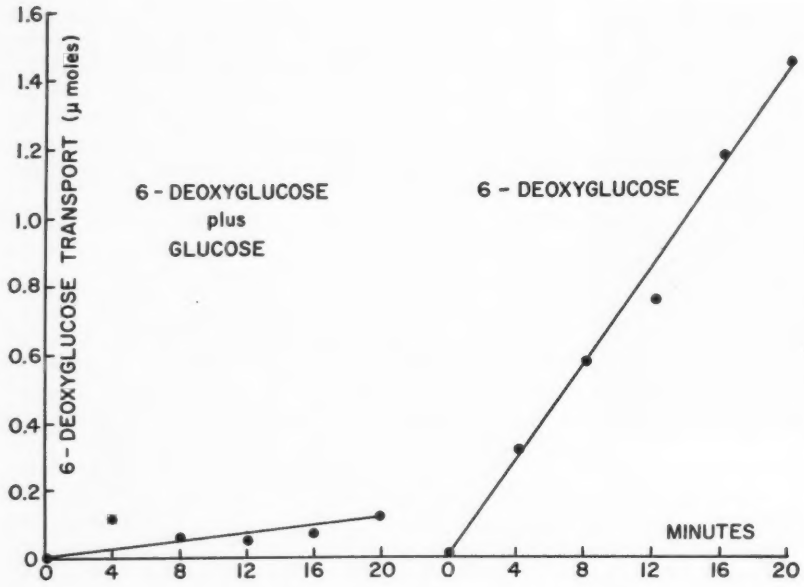


FIG. 5 Effect of glucose (12 mM) on transport of 6-deoxyglucose (1 mM).

TABLE 1. *Effect of Substances on D-Galactose Transport*

D-Galactose concentration = 5 mM

Test Substance	Concentration mM	Inhibition of Galactose Transport* %
None	—	9
Glycine	5	6
L-Methionine	5	10
D-Xylose	25	7
D-Mannose	25	−4
α-Methyl-D-glucoside	25	95
α-Methyl-D-glucoside	5	51
D-Glucose	5	42
1-Deoxy-D-glucose	5	37
3-O-Methyl-D-glucose	25	59
6-Deoxy-D-glucose	5	47

* Average of three determinations. Inhibitions of less than 20% are not considered significant in this system.

tested for mutual competitive inhibition by the method of Crane and Wilson (39). A simplified experimental design is presented in figure 5 in which two time periods were studied with the same segment of intestine, first with 6-deoxyglucose plus inhibitor (glucose) and second, with 6-deoxyglucose alone. Glucose inhibited the transport of 6-deoxyglucose and the inhibition was reversible. With glucose as the test sugar inhibition was observed with both 1-deoxyglucose and α-methylglucoside. Table 1 gives a summary of the effect of a variety of transported sugars on the transport of galactose by this method (40). In every case examined (9 different sugar combinations), the presence of one transported sugar inhibited the transport of the second. These data strongly indicate that a common pathway is involved in sugar transport. This conclusion allows one to delineate the minimal structural requirement for the sugar transport system by hamster intestine from the experiments on specificity (fig. 6).

A few comments on various mechanisms which have been proposed for sugar transport may now be made. The mutarotation theory of glucose absorption (41) is unlikely since two compounds which cannot undergo mutarotation (1-deoxyglucose and α-methylglucoside) were transported and competed with glucose. Moreover, phosphorylation-dephosphorylation as a mechanism for transport has been ruled out for all hydroxyl groups except that at carbon 2. Crane and Krane (33) showed that the oxygen of the hydroxyl group at carbon 2 did not exchange with the oxygen in water during transport, thus eliminating certain chemical reactions such as dehydration and rehydration as the mechanism of transport. While the present data cannot be used to elucidate the mechanism involved in active transport it is useful in excluding certain hypotheses and in stimulating further research.

AMINO ACID ABSORPTION

In adult animals protein molecules are probably completely hydrolyzed to free amino acids before any appreciable absorption occurs. These free amino acids are absorbed by the intestine and appear in the portal vein blood (42). Prior to 1950 evidence for selective absorption of amino acids was inconclusive. On the one hand Höber and Höber (43) showed that certain amino acids were absorbed faster than polyhydric alcohols of equivalent molecular size and that unlike these alcohols the absorption rates were not proportional to concentration in the lumen. They therefore concluded that some type of selective absorption process was involved. On the other hand, the rate of absorption of various amino

acids in the chick varied inversely with the molal volume of the compound (44) and no difference in absorption rate could be established between the two stereoisomers (45).

The first unequivocal demonstration of an active transport process for L-amino acids was the work of Wiseman and his collaborators (46, 47), performed in anesthetized rats, Thiry-Vella loops of unanesthetized dogs (48), and rat intestine *in vitro* (49). When a DL-amino acid was placed on both sides of the intestinal wall *in vitro*, the L-isomer was transported against a concentration gradient while the D-isomer was unaffected. The results of such an experiment are shown in figure 7 for DL-alanine. The transport process was apparently specific for monoamino, monocarboxylic amino acids; the diamino and dicarboxylic amino acids were not transported against concentration gradients (11, 49, 50). The amino acids that were actively transported shared a common pathway within this group (14, 51).

Agar, Hird and Sidhu (12) introduced a simplified *in vitro* method by measuring the accumulation of amino acids by small rings of intestinal tissue. With this method they demonstrated that the accumulation of amino acids by the tissue exhibited the same specificity observed with other methods.

Further studies on the structural requirements for active transport has recently been made with a number of aromatic amino acids and their derivatives with the everted sac technique. Figure 8 shows the aromatic substituents on the β-carbon atom of these amino acids. Para- and meta-tyrosine were transported while the ortho-derivative was not (52). Mono but not di substituted tyrosines were active in this system. These observations suggest that the transport system was sensitive to the nature of the side chain of the amino acid, although the precise requirements can not be defined at present.

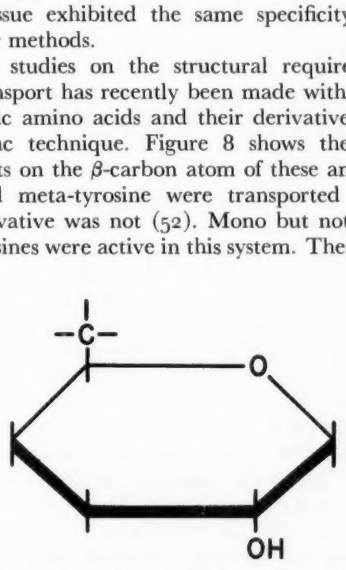


FIG. 6. Structural requirement for sugar transport by hamster intestine.

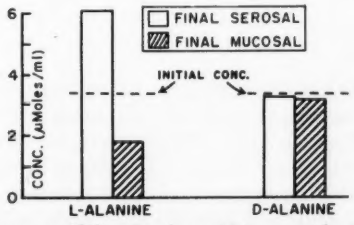


FIG. 7. Stereospecificity of amino acid transport by the intestine. DL-alanine was placed on both sides of isolated rat small intestine. Data were recalculated from Wiseman (11).

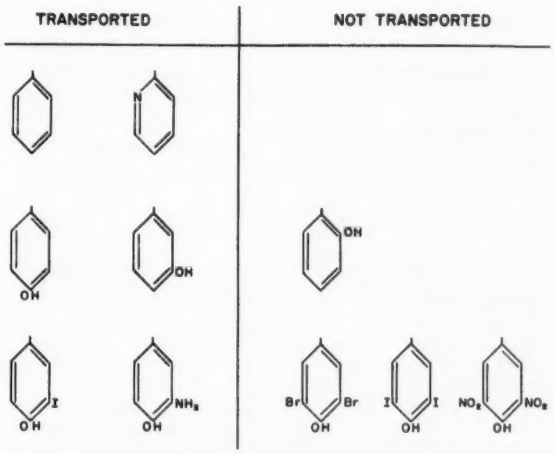


FIG. 8. Transport of some analogues of phenylalanine and tyrosine. The structure given in the figure represents the aromatic substituent on the β-carbon of the amino acid.

A number of derivatives of histidine were also tested for transport (E. C. C. Lin and T. H. Wilson, unpublished observations). The acetylation of the α-amino group of histidine yielded an inactive compound (fig. 9). In addition urocanic acid was also not transported. Further evidence implicating the amino group for transport is provided by the apparent requirement of pyridoxal for this process. Jacobs and his collaborators (53, 54) and recently Suda and his collaborators (55) have shown that pyridoxine deficient rats had reduced capacity to absorb amino acids and this impairment could be rapidly reversed by intravenous injection of pyridoxal or pyridoxine. Additional evidence was recently obtained with pyridoxine deficient hamsters which absorbed L-histidine at a reduced rate with no concomitant effect on galactose transport (E. C. C. Lin and T. H. Wilson, unpublished observations). The importance of the carboxyl group for transport was suggested by the fact that the methyl ester of L-histidine was not transported against a concentration gradient.

The fact that this absorption process was specific for L-amino acids suggested that the transport system interacted with at least 3 groupings on the asymmetric carbon atom. The study of the histidine derivatives suggested that both the amino and carboxyl groups were involved in such an interaction. A comparison between the rates of transport of L-alanine and α-amino isobutyric acid (fig. 10) indicated that the hydrogen of the alpha carbon atom was important[5] and could not be replaced by a methyl group without a great reduction of activity of

[5] A similar set of comparisons was previously carried out by Paine and Heinz (56) with Ehrlich carcinoma cells. In these cells L-alanine and α-amino isobutyric acid were transported at similar rates.

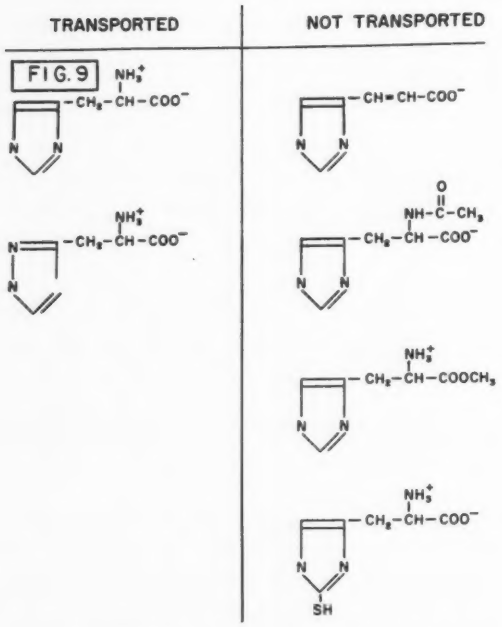


FIG. 9. Transport of some analogues of L-histidine.

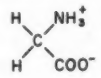
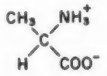
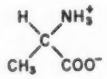
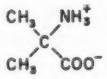
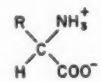
AMINO ACID	STRUCTURE	TRANSPORT RATE (μ moles/100mg tissue)
FIG. 10 GLYCINE		9.1
L-ALANINE		10.2
D-ALANINE		0
α-AMINO-ISOBUTYRIC ACID		1.4
MONO AMINO MONOCARBOXYL L-AMINO ACIDS		

FIG. 10. The effect of substituents of the α-carbon of amino acids on active transport.

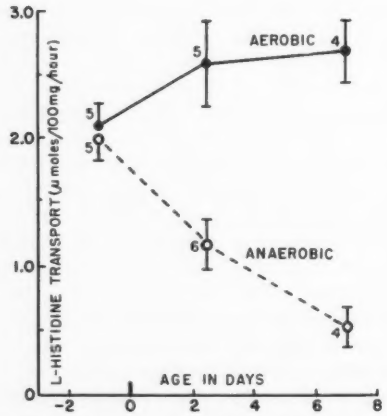


FIG. 11. The effect of age on intestinal transport of histidine under aerobic and anaerobic conditions. Sacs of everted intestine were prepared from foetal and young rabbits.

the compound. With the implication of the amino, carboxyl and alpha hydrogen, it was not necessary to postulate additional interaction with the side chain. In fact, it was found that a number of neutral amino acids were transported and competed with each other. The substituents on the side chain could be a hydrogen as in glycine (49), an indole ring as in tryptophan (R. P. Spencer and A. Samiy, personal communication) or groups not naturally occurring as in the analogues shown in figures 8 and 9.

EFFECT OF OXYGENATION ON TRANSPORT

Movement of compounds against chemical potentials clearly would require the expenditure of metabolic energy. It was not surprising, therefore, that anaerobic conditions prohibited the active transport of sugars and amino acids (10, 13). However, it has been found that the foetal intestine was able to transport substances at rapid rates under nitrogen provided there was sufficient anaerobic glycolysis (fig. 11). Intestinal sacs from foetal rabbits transported histidine equally well aerobically and anaerobically. During the first week of life, this capacity for anaerobic transport was rapidly lost, and in 3 weeks was virtually reduced to zero (57). This experiment also demonstrated that the rabbit was born with a fully developed intestinal transport system for histidine.

CONCLUSION

In conclusion, this discussion has attempted to review some of the recent work on intestinal absorption of sugars and amino acids. It has shown two types of experiments for which the *in vitro* method is particularly suited: firstly, the study of transport specificity of sugars and amino acids and secondly, the study of energy requirements for active transport by the small intestine.

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Digestion and absorption of fat after a normal meal[1]

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FAT ABSORPTION REFERS to the sequence of events from the digestion of dietary fat to its appearance in the circulation. This process is usually considered in three phases: the first is the intraluminar or digestive phase during which the fat is modified physically and chemically prior to absorption; the second is the cellular or absorptive phase when the digested material passes into the mucosa and is prepared for the final distributive phase during which the lipid is transported from the mucosa to the other tissues.

In spite of intensive investigation, these three phases are incompletely understood. It has been claimed on the one hand that in normal digestion lipolysis is complete (1), and on the other that it is partial (2). A third school holds views intermediate between these two extremes (3, 4). Controversy surrounding our knowledge of the mucosal phase is even greater. Similarly, the transport of the absorbed fat is still a matter of conjecture, although the weight of evidence indicates that the major pathway for long chain fats is by way of the lymphatics.

The most important reason for the confusion is that suitable methods for lipid analysis were not available until recently. Attempts to overcome this lack of methodology frequently led to an unphysiological approach in experimental design. The combined use of silicic acid column chromatography, radioactive tracer techniques and gas liquid phase chromatography (GLC) now make it possible to analyze in minute detail the changes in lipid composition of very small samples of tissue from experimental animals following the voluntary digestion of a normal meal.

As a preliminary to further experiments with animals and human subjects, the following study was performed with rats to observe the digestion and absorption of a normal meal into which had been incorporated a small quantity of C^{14}-labeled tripalmitin.

METHODS

Experimental. A detailed description of the feeding procedure is presented because it differs in many ways from methods used in the majority of previous studies. The procedures employed approximated the normal state, which was the object of this study.

Sixty young rats were maintained on Purina rat chow, and trained to eat 4 grams in 10 minutes, which was twice the amount fed on the day of the experiment. The animals continued their normal weight gain on this regimen.

In previous experiments, changes in mucosal phospholipids had been observed to continue for 12 hours after feeding. Therefore, on the eighth day, the animals were fed the test meal after an 18-hour fast. Water was allowed ad libitum. The rats were killed by decapitation and exsanguination.

The test meal consisted of ground Purina chow into which C^{14}-carboxy-labeled tripalmitin dissolved in solvent had been blended. The solvent was removed by drying and the chow blended further to ensure even distribution of the labeled fat. Measurement of radioactivity in three aliquots of the meal confirmed the uniformity of the mixture. The test meal was weighed into aluminum feeding dishes in 2-gram amounts and a small volume of water was added. When the water dried, a hard cake was formed which was similar in consistency to the usual chow. Each meal was calculated to contain 28 mg of the labeled tripalmitin in 138 mg of total lipid content.

Four groups of 12 animals were killed at 1, 3, 4 and 8 hours after feeding. The remaining 12 animals were killed in the fasting state. Clamps were applied at the pyloro-duodenal junction and at the lower end of the ileum. The stomach and small intestine were then quickly removed. The stomach was cut along the greater curvature, the gastric bolus removed, and the remainder of its contents obtained by gentle washing of the mucosal surface with normal saline. The intestinal mucosa from the two pieces of intestine was extruded by exerting gentle pressure on the serosal side of the gut in such a manner as to prevent gross contamination with underlying tissue. The liver was removed and cut up into small pieces. All specimens were pooled with those obtained from animals in the same time group. The removal and collection of the specimens from each animal took less than 5 minutes, by which time they were all frozen with dry ice. The blood was heparinized, chilled

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[3] Fellow, China Medical Board of New York, Inc.

and centrifuged at 3000 rpm for 30 minutes, and the plasma removed.

Analysis. The nine specimens from the five groups of animals (gastric contents, proximal and distal intestinal contents, proximal and distal mucosa, plasma, liver, heart and adipose tissue) were lyophylized, homogenized in chloroform:methanol (2:1 v:v) and extracted for 5 hours with 24 times (w:v) the chloroform:methanol solution. The filtered extracts were then washed with an equal volume of normal saline.

Aliquots from each lipid extract were weighed, the radioactivity measured and the major lipid classes separated by silicic acid column chromatography. The silicic acid (100–200 Mesh) was acid-washed and dried *in vacuo* at 50°C for 2 hours (M. Horning, personal communication). The following fractions were eluted in order from a single silicic acid column with increasing concentrations of benzene in hexane, benzene, chloroform, and increasing concentrations of methanol in chloroform: cholesterol esters, triglycerides, free cholesterol and fatty acids, diglycerides, monoglycerides, 'fraction VI' (this contains phosphatidic acid and also unidentified contaminants), cardiolipins, 'fraction VIII' (unidentified, possibly plasmalogens), cephalins (phosphatidylserine and ethanolamine), phosphoinositide and lecithin (the separation of these two peaks is incomplete, and up to 5% contamination of the lecithin fraction with phosphoinositide has been observed), sphingomyelin, and lysolecithin (5).

The fatty acids were found to be mainly in the cholesterol fraction (at least 80%) with a small quantity contaminating the triglycerides. These were extracted from the two fractions using Borgstrom's technique (6).

The nature and purity of the individual fractions were confirmed by applying the following methods of analysis, where appropriate: ratios of glycerol (7), fatty acids (8), ester bond (9), phosphorus (10) and amino nitrogen. In addition, paper chromatographic analysis of individual fractions against pure standards as well as differential staining was carried out.

Radioactivity. An aliquot of each fraction was added to a standard toluene-PPO (2,5-diphenyloxazole) solution and the C^{14} radioactivity measured in a liquid scintillation counter (Tri-Carb). The results have been expressed as specific activity (SA), i.e. c.p.m./mg.

TABLE 1. *Fatty Acid Composition of Test Meal*

Fatty Acid	%	Fatty Acid	%
14:0	2	18:0	6
16:0	52	18:1	23
16:1	1	18:2	16

TABLE 2. *Total Glycerides and Free Fatty Acids in Gastric Contents*

Time, hr.	Weight, mg	Specific Activity, c.p.m./mg × 10^{-3}
0	4	0
1	737	45
3	460	46
4	192	43
8	16	9

TABLE 3. *Total Glycerides and Free Fatty Acids in Intestinal Lumen and Mucosa, and Plasma*

Time, hr.	Weight, mg		Weight, mg/10 ml Plasma
	Lumen	Mucosa	
0	16	15	1.8
1	81	51	2.6
3	71	55	5.7
4	68	56	
8	44	52	1.7

TABLE 4. *Total Glycerides and Free Fatty Acids in Proximal and Distal Intestinal Lumen and Mucosa*

Time, hr.	Weight, mg			
	Proximal		Distal	
	Lumen	Mucosa	Lumen	Mucosa
0	3.9*	9.5	13.8*	12.4
1	51.6	25.3	29.6	26.4
3	25.8	24.4	45.4	29.4
4	19.7	20.8	48.1	35.7
8	14.0	23.0	29.7	29.0

* Includes weight of cholesterol ester and free cholesterol.

Gas-liquid phase chromatography. Aliquots of each fraction were hydrolyzed and methylated in methanol-H_2SO_4 in a teflon-stoppered tube for gas chromatographic analysis. The analysis was performed using a 6-foot glass column packed with 80–100 mesh Chromosorb-W coated with diethylene glycol succinate (17%). The column temperature was maintained at 200°C, and an argon-tritium ionization mass detector was used with a flow rate of 100 cc argon/min.

RESULTS

A vast amount of data accrued from this experiment. The majority of this data is included here, but for simplicity, and since the radioactivity findings and the gas-liquid phase chromatographic results are complementary in many instances, the latter are omitted except where their inclusion provides additional information.

Lipids of the meal. The 24 gm of powdered rat chow fed to each group of 12 animals contained 1656 mg, of which the mean intake, correcting for the meal not eaten, was 1584 mg. Eight per cent of the lipid was phospholipid, and 2.6% cholesterol esters and cholesterol in a proportion of 3:1. The SA of the neutral lipids was 46,430 c.p.m./mg. The fatty acid pattern of the meal is shown in table 1.

Lipids of the gastric contents. The weights and SA of the lipids in the stomach are given in table 2. The SA and GLC analyses showed no specificity in the way in which the dietary fat was discharged into the intestinal lumen during the first 4 hours after the meal.

Lipids of the intestinal lumen. The glyceride and free

fatty acid (FFA) content of the intestinal lumen remained relatively constant at 1, 3 and 4 hours after the meal (table 3); it was 5.1, 4.5 and 4.3% of the lipids eaten. The amounts and character of these lipids differed between the upper and lower halves of the intestine (tables 4, 5 and 6). For example, the majority of the lipid in the lumen was present in the proximal half at 1 hour, but in the distal half at 3, 4 and 8 hours. Hydrolysis in the proximal lumen was more complete than in the distal. Contrary to this, the SA of the different lipid classes was usually greater distally. The greater radioactivity was found in those fractions with a high palmitic acid content, as determined by GLC analysis.

The phospholipids of the intestinal lumen are shown in tables 7 and 8. The only phospholipid fraction with radioactivity was 'fraction VI' which is a mixture containing phosphatidic acid. There was more lecithin than lysolecithin within the proximal lumen, apart from the 3-hour results, but the reverse was true in the two observations made on the distal contents.

Mucosal lipids. After the meal, the weight of the glycerides and FFA of the intestinal mucosa was constant (table 3). Details of the weights and specific activities of the glycerides and FFA of the proximal and distal mucosa are given in tables 4, 5 and 6. Following the meal, the majority of the lipid in the mucosa was in the form of FFA. The SA of each fraction was highest at 1 hour; however there was a marked reduction in the SA in the material in the mucosa, compared with that in the lumen, at 3 and 4 hours in both the proximal and distal halves.

The SA and GLC relationships of the fatty acids in the FFA and triglycerides of the proximal mucosa are shown in tables 9 and 10. The FFA had a higher proportion of unsaturated material and lower stearic acid content than the triglycerides. Table 11 shows the fatty acid pattern in the triglycerides of the distal mucosa. The palmitic acid content was similar to that of the triglycerides of the proximal mucosa; however the stearic acid content was considerably lower and the unsaturated fatty acid content greatly increased. There was a significant absence of linolenic (C-18:3) in the distal mucosa.

Details of the phospholipids of the proximal and distal mucosa are shown in tables 7 and 8. The results show a reduction in the amount of cephalin at 1, 3 and 4 hours, a considerable increase in lecithin at 1 hour, and an absence or small increase of specific activity of all the phospholipids when compared with the mucosal glyc-

TABLE 5. *Glycerides and Free Fatty Acids in Proximal Intestine*

Time, hr.	Lumen				Mucosa			
	Tri-	Di-	Mono-	FFA	Tri-	Di-	Mono-	FFA
0 Wt.*	3.9				2.1	1.8	2.7	0.7
SA†	0				0	0	0	0
1 Wt.	5.7	7.9	2.2	35.8	4.2	3.1	4.4	13.6
SA	15.6	33.5	34.4	42.6	30.7	42.6	18.5	43.4
3 Wt.	1.0	4.4	0.3	19.1	5.9	3.8	0.9	14.2
SA	0.8	10.0	8.1	82.5	16.1	5.2	2.4	19.8
4 Wt.	1.6	4.1	0.2	14.5	2.4	4.4	0.5	13.5
SA	1.3	6.7	3.5	65.1	16.4	3.5	3.3	15.6
8 Wt.	1.6	3.8	0.7	7.9	1.6	4.1	2.9	14.4
SA	0.3	1.4	10.9	19.2	1.2	1.1	3.7	11.1

* Wt. = weight in mg. † SA = c.p.m./mg × 10^{-3}

TABLE 6. *Glycerides and Free Fatty Acids in Distal Intestine*

Time, hr.	Lumen				Mucosa			
	Tri-	Di-	Mono-	FFA	Tri-	Di-	Mono-	FFA
0 Wt.*	13.8				3.0	3.2	0	0
SA†	0				0	0	0	0
1 Wt.	9.4	6.0	2.1	12.1	5.4	3.8	3.95	13.8
SA	129.5	25.3	3.8	54.5	44.6	9.5	1.8	24.0
3 Wt.	19.3	7.0	4.2	14.9	4.1	3.8	2.5	19.0
SA	110.5	10.0	4.1	88.2	16.5	1.8	0.1	18.7
4 Wt.	12.2	7.8		28.1	4.5	4.6	3.5	23.1
SA	48.0	7.0		94.0	5.3	1.7	5.9	21.7
8 Wt.	7.4	10.1	5.4	6.8	12.8	6.4	6.7	12.8
SA	18.0	3.4	1.9	54.5	8.3	0.8	1.3	12.3

Monoglyceride sometimes contaminated with extremely small amounts of unidentified phospholipid. * Wt. = weight in mg. † SA = c.p.m./mg × 10^{-3}.

TABLE 7. *Phospholipids in Proximal Intestine*

Time, hr.	Lumen				Mucosa			
	'Peak VI'	Cephalin	Lecithin	Lysolec.	'Peak VI'	Cephalin	Lecithin	Lysolec.
0 Wt.*	2.6				2.3	3.5	5.8	0.8
SA†	0				0	0	0	0
1 Wt.	1.2	2.7	2.2	0.30	0.6	3.0	16.7	0.8
SA	8.0	0	0	0	trace	0	5.6	0
3 Wt.	1.3	0.81	0.3	1.02	2.0	2.6	3.0	1.0
SA	5.2	0	0	0	9.8	0	0.8	0
4 Wt.	2.0	1.6	1.3	0.4	2.1	2.1	2.6	0.8
SA	2.7	0	0	0	8.9	0	0.7	0
8 Wt.	1.5	0.8	1.8	0.4	1.0	3.6	6.4	1.1
SA	3.4	trace	trace	trace	4.7	0.4	3.4	0

* Wt. = weight in mg. † SA = c.p.m./mg × 10^{-3}.

TABLE 8. *Phospholipids in Distal Intestine*

Time, hr.	Lumen				Mucosa			
	'Peak VI'	Cephalin	Lecithin	Lysolec.	'Peak VI'	Cephalin	Lecithin	Lysolec.
0 Wt.*	6.32				1.6	2.25	0.8	0.3
SA†	0				0	0	0	0
1 Wt.	3.0	2.9	1.0	2.5	1.4	1.0	21.3	1.0
SA	5.4	0	0	0	1.8	0	0.8	0
3 Wt.	5.6	5.35			2.6	1.3	2.1	0.75
SA	1.4	0.5			4.3	0	0.6	0
4 Wt.		6.1	0.9	2.0	2.5	0.8	0.6	2.1
SA		0.5	0	0	0.9	0	trace	0
8 Wt.	21.4				0.7	6.4	7.2	0.1
SA	0.3				0	0.1	1.3	0

* Wt. = weight in mg. † SA = c.p.m./mg × 10^{-3}.

TABLE 9. *Fatty Acid Composition of Free Fatty Acids in Proximal Intestinal Mucosa*

Time, hr.	Weight, mg	SA, c.p.m/mg × 10^{-3}	Fatty Acids, %					
			16:0	16:1	18:0	18:1	18:2	20:4
0	0.70	0	24	0	27	9	29	11
1	13.6	43.4	70	2	9	13	6	0
3	14.2	19.8	25	0	12	19	38	7
4	13.5	15.6	36	0	14	14	30	6
8	14.4	11.1	34	0	24	4	38	0

TABLE 10. *Fatty Acid Composition of Triglycerides in Proximal Intestinal Mucosa*

Time, hr.	Weight, mg	SA, c.p.m/mg × 10^{-3}	Fatty Acids, %						
			16:0	16:1	18:0	18:1	18:2	18:3	20:4
0	2.1	0	7	3	15	15	23	7	37
1	4.2	30.7	70	0	23	6	trace	trace	0
3	5.9	16.1	46	2	41	4	2	5	trace
4	2.4	16.4	51	0	30	4	10	1	4
8	1.2	1.6	36	10	19	15	13	0	7

TABLE 11. *Fatty Acid Composition of Triglycerides in Distal Intestinal Mucosa*

Time, hr.	Weight, mg	SA, c.p.m/mg × 10^{-3}	Fatty Acids, %					
			16:0	16:1	18:0	18:1	18:2	20:4
0	3.0	0	55	7	5	6	9	18
1	5.4	44.6	66	0	10	7	10	7
3	4.1	16.5	43	0	4	22	31	trace
4	4.5	5.3	24	23	9	17	19	8
8	12.8	8.3	49	3	7	21	20	trace

erides and FFA. 'Peak VI,' which contains phosphatidic acid, had the highest SA. Since this fraction is known to be a mixture, the specific activity if limited to a single compound would be considerably increased. The cardiolipins, 'fraction VIII,' and sphingomyelin showed no change in weight and no specific activity during the experiment.

Plasma lipids. For comparative purposes the results in table 12 have been expressed per 10 ml. The small quantities of glycerides and FFA in plasma leave much to be desired in these results, and interpretation must be guarded. A slight error in the quantitation of FFA could account for a marked reduction in its specific activity. However, a consistent increase in FFA radioactivity was observed. The highest levels of specific activity in the triglyceride fraction occurred at 3 hours, which was coincident with a measurable increase in weight. The reduction in the amount of cephalin following feeding and the threefold increase of the lecithin at 3, 4 and 8 hours is noteworthy. The SA of lecithin was small but increased after the third hour. Detectable amounts of 'fraction VI' were present only in the fasting state. Similar observations have been made on human plasma.

Contrary to the findings in the glyceride and FFA fractions of the plasma, the phospholipids remained surprisingly constant in amount for 8 hours following the test meal, with the exception of lecithin (table 12). The small but gradual increase in plasma lecithin weight and radioactivity was not coincident with a significant increase in the proportion of palmitic acid (as measured by GLC) in this moiety (table 15).

Liver Lipids. The weights and SA of the various major lipid fractions are given in table 13. The decrease in the total glycerides, especially at 3 and 4 hours, was due to the decrease in triglycerides and monoglycerides. The interpretation of the specific activity of the FFA is not possible, as these were not separated from the free cholesterol (apart from the fasting level, when there were 98 mg cholesterol) and are expressed in relationship to their combined weight; their activity would be greater than that shown. Compared to the fasting values, 'Peak VI' showed an increase in weight at 1, 3 and 4 hours, with a gradually increasing specific activity which was highest at 8 hours. Similar changes occurred in the lysolecithin fraction. Unlike the cephalin of the intestinal mucosa and the plasma, an increased weight in liver cephalin was observed at 3, 4 and 8 hours. In contrast

TABLE 12. *Plasma Lipids*

Time, hr.	Glyceride			FFA and Chol.	'Peak VI'	Cephalin	Lecithin	Lysolecithin
	Tri-	Di-	Mono-					
0 Wt.*	1.0	0.6	0.2	0.1	0.7	2.0	3.8	1.0
SA†	0	0	0	0	0	0	0	0
1 Wt.	1.1	0.8	0.7	0.7	0	0.9	3.2	2.0
SA	10.7	0	0	9.1	0	0	0	0
3 Wt.	3.7	1.2	0.8	0.2	0	0.8	10.8	1.4
SA	21.1	4.6	0	101.0	0	0	0.9	0
4 Wt.	lost	lost	lost	lost	lost	1.0	8.1	2.8
SA						0	1.2	0
8 Wt.	0.4	1.1	0.4	0.3	0	1.2	10.4	1.7
SA	1.3	4.7	1.4	134.0	0	0	1.9	0

* Wt. = weight in mg/10 ml. † SA = c.p.m./mg × 10^{-3}.

TABLE 13. *Liver Lipids*

Time, hr.	Glyceride			FFA and Chol.	'Peak VI'	Cephalin	Lecithin	Lysolecithin
	Tri-	Di-	Mono-					
0 Wt.*	74.0	9.2	38.0	116.0	20.0	308.2	684.4	7.6
SA†	0	0	0	0	0	0	0	0
1 Wt.	56.0	3.2	21.2	104.0	44.0	303.2	682.6	21.6
SA	4.3	0	0.3	0.6	0.6	0.3	0.6	0.2
3 Wt.	32.4	25.6	14.4	134.4	41.2	364.0	631.0	15.2
SA	1.4	6.3	10.9	3.2	1.6	1.8	3.6	1.5
4 Wt.	16.8	18.4	19.6	130.4	40.9	341.2	607.0	16.0
SA	21.6	5.2	6.0	2.8	1.6	1.8	3.9	1.5
8 Wt.	41.8	10.7	36.6	131.0	13.9	356.6	667.0	11.3
SA	3.7	1.6	2.4	2.2	6.4	3.0	3.9	3.8

* Wt. = weight in mg. † SA = c.p.m./mg × 10^{-3}.

to the mucosa and plasma, however, an increase in liver radioactivity from 1 hour was observed. The lecithin weight fell slightly in the 3 and 4 hour specimens, and then began to rise in the 8 hour fraction. The 3, 4 and 8 hour lecithin SA remained quite constant.

DISCUSSION

The influence of the test meal. It is felt to be of utmost importance that fat absorption should be studied during the assimilation of a normal meal. The gastric bolus which is not only related to the nutrients but also to the roughage of the meal is known to affect the rate of gastric emptying (11). Also it is reasonable to expect that the composition and quantity as well as the rate of passage of fat into the duodenum will influence fat digestion and absorption. In fact it has been shown that the presence of glycerides and different fatty acids influence the rate and extent of fat absorption (12); the presence of certain amino acids accelerate the hydrolysis of olive oil by pancreatic lipase (13), and tolerance curves after a meal of I^{131}-triolein are altered by the simultaneous administration of glucose or casein (14). Thus, observations from experiments in which fat alone is fed provides information pertinent only to these experimental conditions which are not necessarily physiological. Similarly, tube feeding avoids interaction of dietary constituents and the usual sequence of physiological events, and must be considered abnormal.

Not only is the character of the test meal important, but also the effect of one meal upon another (15) which, together with variations in the nonlipid components, may have a similarly important bearing on changes in fat absorption and metabolism which in turn may have pathological significance.

Gastric function. The role of the stomach in fat digestion and absorption has received little attention. It is more than probable that it influences the rate of fat absorption by regulating the flow of lipid into the small intestine. The data presented here shows that the gastric contents contained 52, 32 and 14% of the neutral lipids in the meal at 1, 3 and 4 hours, respectively. Yet, in the same period, the contents of the intestinal lumen remained constant at 5.1, 4.5 and 4.3%, though the distribution of this fat was not consistent between the upper and lower halves of the intestine. However the lipid content of the upper and lower mucosa was constant. Thus it is possible that the degree of saturation of the mucosa with lipid, a feature which is dependent on the removal of lipid to the lymph and blood, may be of importance in influencing the rate of gastric emptying. In view of the effect of dietary components on gastric emptying, this suggestion of the role of the stomach in fat absorption once again stresses the importance of the character of the test meal.

Digestion and the luminal contents. The examination of the glycerides and fatty acids of the intestinal lumen has been commonly used as an index of digestion (16, 17). The degree of complete hydrolysis, as indicated by the amount of fatty acid in rat intestinal contents, has been reported to vary between 17.6 and 42.5% after the administration of 2 ml olive oil (18); 0.75 ml corn oil (19); and 1.5 ml partially hydrogenated vegetable oil (20). The results reported here show a similar degree of hydrolysis obtained at comparable time intervals when the hydrolysis values in the upper and lower halves of the intestine were combined and corrected for differences in the quantity of lipid in the two halves. Differences between these results and those of others can probably be attributed to the more physiological test meal used.

The greater degree of hydrolysis observed in the proximal compared to the distal intestine supports the necessity of separating the upper and lower halves in this type of study. The products of protein digestion (13) may augment the influence of bile and pancreatic lipase in the proximal lumen to account in part for this improved digestion. The greater degree of hydrolysis in the proximal half, especially at 1 hour, and the accepted importance of this portion of the intestine in the early stages of absorption, may be indicative of a functional difference between the upper and lower intestine in fat absorption.

The SA of the FFA at 1 hour closely resembled that of the test meal. The increased radioactivity at 3 and 4 hours indicates specific absorption of nonlabeled fatty acids with an accumulation of C^{14}-labeled palmitic acid. Together with these findings, selective absorption of nonlabeled fatty acids is supported by the consistently lower radioactivity of the proximal mucosa and the increased radioactivity of the fatty acids in the distal lumen up to 4 hours. Additional GLC analysis of the FFA of the lumen also showed a relative accumulation of palmitic acid. This must have been due to more rapid absorption of other fatty acids in the meal under these conditions.

It is possible that differences in rates of hydrolysis of certain triglycerides together with reduced absorption of C^{14}-labeled tripalmitin accounts for the variations in the radioactivity of triglyceride in the proximal and distal lumen. However, Borgstrom (19, 21) has presented evidence for the formation of new ester bonds under conditions resembling those in the small intestine. An accumulation of labeled fatty acid distally could account for the synthesis of triglyceride with high specific activity. Also the equilibrium which exists between unabsorbed fatty acids and the glycerides in the lumen would account for further accumulation of labeled fatty acid in the glycerides.

The marked degree of hydrolysis in the proximal lumen and the greater proportion of glycerides in the distal lumen suggests that after 1 hour the majority of lipid is hydrolyzed completely prior to absorption, since it is unlikely that exogenous glycerol could play a part in the resynthesis of glycerides in the distal lumen (22).

Rate of absorption. The plasma triglyceride, the chylomicron counts and the turbidity of the chyle reach a maximum at 2–4 hours after a fatty meal. The peak

plasma radioactivity occurs at a similar time after I^{131}-labeled triolein ingestion (14). These observations have led to the assumption that maximum rate of fat absorption also occurs at this time. Fat absorption continues for a number of hours; however the results in table 14 show that within 1 hour, 42% of the fat ingested could not be accounted for in the stomach or small intestine. This was the fastest rate of absorption in the time intervals studied. Evidence for highly significant absorption of fat in this early period was confirmed by the peak radioactivity of the neutral lipids and FFA in the intestinal mucosa and the considerable incorporation of C^{14}-labeled lipid into the plasma, liver, myocardium and adipose tissue at one hour. Borgstrom (12) studying the intestinal absorption of different fat mixtures in rats observed 58–82% absorption in the first hour. Thus the greatest plasma glyceride weight and radioactivity observed at 3 hours (table 12) does not reflect the time of maximal absorption but more probably represents a time when the tissue uptake of lipid is reduced, while absorption continues. Early and elevated plasma neutral lipid and I^{131}-triolein levels after a fat meal, associated with a decreased rate of removal of lipid from the plasma to the peripheral tissues have been reported in dogs and man (14). The increased turbidity of the chyle which occurs after similar delay might well be due to a reduction in lymph flow. The maximum flow of chyle has been reported to precede the time of the greatest lipid concentration (23).

Absorption and the changes in mucosal lipids. The absorption of fat was thought to occur by solution as soaps resulting from complete hydrolysis (1), by the entry of small lipid particles through microscopic canals (2) and possibly by changes in the character of lipid by phosphorylation (24). Examination of the lipids of the intestinal mucosa shows not only fat in the form in which it is absorbed from the lumen but also a large amount of material which may be undergoing change prior to transport. Dawson and Isselbacher (25) have confirmed that triglyceride synthesis occurs in the intestinal mucosa. However, certain lipids may be more difficult to utilize or transport than others, and could accumulate to a relative extent within the mucosa. The changes observed in the intestinal mucosa must therefore be interpreted cautiously.

In view of the extent of hydrolysis observed (tables 5, 6), particularly in the proximal lumen, it seems probable that under the conditions studied here the majority of dietary fat was completely hydrolyzed prior to absorption. However, only at 1 hour in the proximal half of the intestine was there a close similarity between the GLC analysis and the specific activity of the luminal and mucosal FFA. The first hour was also the time of highest lipid content in the proximal lumen and the most rapid rate of absorption, suggesting that the upper intestine may have the dominant role in the early stage of absorption. Although no specificity of absorption is indicated here, the palmitic acid content of both the mucosal triglycerides and FFA was greater than that of

TABLE 14. *Disappearance of Lipid From Lumen of Stomach and Intestine.*

Time, hr	Weight, mg	% weight of lipid fed
0	0	0
1	598	42
3	885	62
4	1156	82
8	1356	96

the test meal. This may mean that an excessive intake of this fatty acid occurred during the first hour. It is of interest to note that during this early phase of absorption in man the highest concentration of intestinal enzymes and bile occurs (26). The concentration of lecithin in the lumen was highest at 1 hour; since there is a high content of lecithin in bile and there was little lecithin in the meal the bile concentration in the proximal intestine was probably maximal at this time. However, a further explanation might be that an unusually rapid uptake of lipids occurred in the early stages of digestion in fasted animals when the mucosal and peripheral tissues have a low lipid content and thus a high capacity for taking up newly absorbed lipid.

The findings in the intestinal lumen during absorption are difficult to explain without suggesting a specificity of fatty acid absorption, and this would account at least in part for the differences between the luminal and mucosal FFA after 1 hour. The variations in the GLC analyses indicate that this specificity of absorption was extremely complex.

The data presented does not allow any conclusion to be drawn as to whether or not monoglycerides and diglycerides as such were absorbed. The differences between the GLC proportions and the radioactivity of the glycerides in the mucosa and in the lumen make it unlikely that they were absorbed to any significant extent. The absorption of intact glycerides may be a more obvious feature following a meal consisting solely of fat or one with an abnormally high fat content.

The inconsistency of the GLC and SA findings in the FFA and glycerides of the mucosa is difficult to understand. The greatest differences occurred between the FFA, monoglycerides and diglycerides, and these differences were more apparent in the SA measurements than in the GLC findings. In fact, at 3 and 4 hours the proportion of palmitic acid (GLC) was higher in the monoglycerides and diglycerides than in either the triglycerides or FFA; however, these lipids may be mainly of endogenous origin. Since the fatty acids and triglycerides of the intestinal mucosa were consistently less radioactive than those of the intestinal lumen, dilution of mucosal lipids by unlabeled lipid must have occurred. Dilution could have been from phospholipid constituents whose radioactivity remained consistently low in the intestinal mucosa throughout absorption; these could have been the source of mucosal mono- and diglycerides of endogenous origin. The changes in the mucosal lecithin, lysolecithin and 'cephalins' would fit in with this scheme. On the other hand, the high SA and

palmitic acid content of the plasma FFA could have resulted from an increased rate of absorption and transport of the fatty acids which were present in excess in the diet, e.g. palmitic acid. This would in turn explain the apparent dilution phenomenon observed. Selectivity of absorption, an endogenous supply of lipid, and a differential release of FFA may be part of a complex mechanism which regulates the fatty acid composition of triglycerides synthesized in the intestinal mucosa. Such regulation may explain in part the lack of influence of dietary lipid on the lipids of chylomicrons (27).

The phospholipids and fat absorption. Sinclair (28) postulated that phospholipids were intermediates in fat absorption. This was supported by the observation that there was an increased turnover of phospholipid during fat absorption (29) and histological changes implicating the phospholipids (30). However, Zilversmit (31) concluded that the turnover of phospholipid was too small to account for the major portion of fats passing through a phospholipid intermediate during absorption. On the basis of later work, however, participation of a small but extremely active individual phospholipid was not excluded (32).

This latter possibility was supported by the observation (tables 7, 8) that 'Peak VI,' a phospholipid peak containing mostly phosphatidic acid, had appreciable radioactivity. Johnson, Tidwell and Bearden (33, 34) have presented evidence that phosphatidic acid may be an important intermediate in fat absorption. The presence of 'Peak VI' with radioactivity in both the intestinal lumen and the intestinal mucosa suggests that 'phosphatidic acid' is important in the resynthesis of triglycerides and perhaps in the transfer of lipid across the mucosal membrane. The virtual absence of labeled fatty acids in the other phospholipids, apart from the lecithin in the proximal intestine at 1 hour, does not support the claim that the other phospholipids are intermediates in the mucosal phase of fat absorption. Apart from the 1-hour sample, the GLC analysis of the mucosal lecithin was fairly constant and resembled that of the plasma (table 15). This agrees with the hypothesis that the phospholipids other than 'Peak VI' may be sources of endogenous lipid. At 1 hour, the increased amount of lecithin with elevated SA in the proximal mucosa was associated with the highest lecithin content found in the lumen. This may explain the difference between the fatty acid composition of the mucosal and plasma lecithin at this time. The absorption of bile lecithin probably accounts for the increased lecithin content, rather than newly synthesized lecithin or dietary lecithin, which was negligible.

TABLE 15. *Fatty Composition of Lecithin in Plasma and Proximal Intestinal Mucosa*

Time, hr.	Fatty Acids, %						
	16:0	16:1	18:0	18:1	18:2	18:3	20:4
0 Mucosa	24	2	23	8	23	trace	20
Plasma	29	6	20	8	19	trace	17
1 Mucosa	47	3	22	8	13	0	6
Plasma	34	3	16	8	25	trace	12
3 Mucosa	37	3	21	8	17	trace	14
Plasma	32	2	18	9	26	trace	13
4 Mucosa	35	4	24	9	15	0	13
Plasma	lost	lost	lost	lost	lost	lost	lost
8 Mucosa	36	1	14	6	25	trace	19
Plasma	33	2	19	9	22	trace	14

TABLE 16. *Cholesterol Esters in Proximal and Distal Intestine, Plasma and Liver*

Time, hr.	Intestine				Plasma	Liver
	Proximal		Distal			
	Lumen	Mucosa	Lumen	Mucosa		
0 Wt.*	3.9‡	2.7	13.8‡	1.0	5.5	51.6
SA†	0	0	0	0	0	0
1 Wt.	3.3	2.3	7.5	0.75	3.9	52.8
SA	5.8	4.4	2.9	0	0	0.8
3 Wt.	1.6	2.2	9.3	3.2	4.9	40.8
SA	2.5	1.1	0.7	0.4	0.7	2.0
4 Wt.	1.6	1.4	13.9	1.8	4.8	30.4
SA	2.4	1.6	1.5	1.3	1.1	2.1
8 Wt.	1.5	1.8	5.7	4.3	4.8	43.6
SA	1.1	1.3	0.5	0.1	3.3	1.6

* Wt. = weight in mg (plasma weight = mg/10 ml).
† SA = c.p.m./mg $\times 10^{-3}$. ‡ Includes weight of neutral lipids.

Cholesterol and cholesteryl esters. The findings in the cholesteryl esters are included for the sake of completeness (table 16). Labeled fatty acids were found in the cholesteryl esters of the intestinal lumen. The maximum SA in the proximal mucosa was at 1 hour. No consistent pattern was found in the fatty acids of the cholesteryl esters of the mucosa, plasma and liver after GLC and radioactivity measurement. The only consistent feature of note was the high content and variety of polyunsaturated fatty acids.

SUMMARY

The experimental design and the resultant data described in the literature on fat absorption must now be re-examined carefully because of recent advances in techniques and analytical methods in lipid chemistry. The analysis of lipids in the past required comparatively large samples. It was difficult to separate completely the fat from other materials, and the methods of analysis were time-consuming, tedious and often inaccurate. There are no longer any problems in separating the lipids from other substances in the lumen, mucosa, blood or lymph. Complete separation of the fat of the lumen or other tissues into the major lipid classes is now routine. In addition, further qualitative and quantitative analysis of the component fatty acids in individual lipids is now possible with GLC as well as chemical measurement of other constituent moieties. Radioactive labeling of the constituents of a test meal make it possible

to follow the transient fate of the fat actually fed and to differentiate it from endogenous lipid.

A study of fat absorption may now be performed under experimental conditions resembling closely the physiological state. This is in direct contrast to earlier experiments where *in vivo* studies had to be carried out with animals subjected to gross procedural trauma; test meals were composed of the single substance being studied and that substance was fed in abnormally large quantities; the absorption of 'raw' fat, quite unlike fat subjected to the gastrointestinal environment, was studied using isolated organs. The influence of other nutrients and roughage on the fat in the diet, as well as other physical and chemical factors to which the fat is normally subjected during passage through the intestine has usually been neglected.

A gastric regulatory mechanism has been postulated which may be related to the degree of saturation of the intestinal mucosa and peripheral tissues with absorbed fat. The absorption of fat was greatest in the first hour, while the maximum rise in plasma triglyceride and radioactivity was at 3 hours. The accumulation of lipid in the plasma must therefore bear a close relationship to the removal of plasma lipid into the tissues. In view of the degree of hydrolysis shown to occur in the upper half of the small intestine and the marked rise observed in mucosal fatty acids, it must be concluded that following a normal meal the majority of dietary fat was completely hydrolyzed. The fatty acid pattern of mucosal triglycerides appeared to be associated with selective absorption, an endogenous supply of lipid and the removal from the mucosa of fatty acids present in excess.

Phosphatidic acid was the only phospholipid into which C^{14}-labeled palmitate was incorporated markedly. An important role in fat absorption must be assigned to this substance. It is possible that this phospholipid has a dual function in fat absorption, as an intermediate in extracellular as well as intracellular processes.

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Mechanism of absorption of vitamin B_{12} [1]

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DR. HOGBEN (1) HAS DISCUSSED 'the first common pathway' in the metabolism of nutrients, namely, passage into the gastrointestinal tract and across the gut mucosa. He pointed out that it has two branches. One of these is a passive road, along which the nutrient moves solely in relation to its quantity, i. e., by diffusion and direct absorption. The other is an active road along which the nutrient moves in an energy-requiring manner.

The mechanism of vitamin B_{12} absorption provides an interesting model of such alternative routes for this first common pathway. A small aliquot of supraphysiologic (pharmacologic) amounts of vitamin B_{12} (i. e., amounts greater than those ordinarily found in the average diet) appears to be absorbed in proportion to the total vitamin B_{12} concentration. This aliquot represents only a tiny percentage of the total amount of vitamin B_{12} presented to the intestine (2, 3). On the other hand, the absorption of physiologic amounts of vitamin B_{12}, perhaps especially when presented in animal protein, is dependent on a specific series of events occurring within the gastrointestinal tract. The rest of our discussion will concern itself with this latter 'physiologic' mechanism.

At first blush it may seem wasteful of us to have spent so much time studying the absorption of vitamin B_{12} since, in a sense, the problem was solved a hundred years ago by Dr. Austin Flint (4) who, in discussing the cause of pernicious anemia, stated "I suspect that in these cases there exists degenerative disease of the glandular tubuli of the stomach . . . fatal anaemia must follow an amount of degenerative disease reducing the amount of gastric juice so far that assimilation of food is rendered wholly inadequate for the wants of the body. I shall be ready to claim the merit of this idea when the difficult and laborious researches of someone have shown it to be correct." And so you see the credit has already been assigned for the fundamental role of gastric juice in the mechanism of absorption of vitamin B_{12}. Nevertheless, it is of interest to pursue this subject further for the pleasure of attempting to dissect out in its entirety the sequence of events involved, and perhaps even, should there be any, to find parallels in the absorption of other nutrients.

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A year ago the status of knowledge with respect to the absorption of vitamin B_{12} in man was reviewed (5), and even more recently the sequential mechanisms in the enhanced absorption of vitamin B_{12} by intrinsic factor in the rat (6). The basis for the 'modern' studies of the mechanism of absorption of vitamin B_{12} was laid some three decades ago (7–10) when it was found that patients with pernicious anemia fed beef muscle remained anemic; but that when this beef muscle was fed with normal human gastric juice, their hematologic derangements were corrected. Thus it appeared that two substances were required to prevent the development of pernicious anemia: *1*) a substance contained in beef muscle or other animal protein which, being supplied from outside the body, was referred to as 'extrinsic factor' and *2*) a substance produced by the normal human stomach which, because it came from the patient himself, was referred to as 'intrinsic factor.' It was believed that extrinsic factor and intrinsic factor combined to form the erythrocyte maturation factor present in liver. Shortly after the isolation of vitamin B_{12} (11, 12) it became evident that both the extrinsic factor and the factor present in liver were vitamin B_{12}. This being so, a major, if not the only function, of intrinsic factor was to facilitate the absorption of vitamin B_{12} (13). With the introduction of Co^{60}-labeled vitamin B_{12} (14) study of this process was greatly accelerated and became possible in patients who were not anemic (15, 16).

The absorption of labeled vitamin B_{12} appears to occur in the ileum in man (17, 18). The site of absorption of vitamin B_{12} may be at different levels of the small intestine in different animal species (19). There is some evidence (20, 21) that a circulating intrinsic factor-like material may be involved in the selective uptake of vitamin B_{12} by the liver and its deposition therein. Considerable further work is needed before this point can be clarified. We shall not discuss it further here except to state that one of the major points which requires settling is whether or not the increased vitamin B_{12} deposition in the liver of rats after the parenteral administration of intrinsic factor and vitamin B_{12} (20, 22) is a physiological event. Deposition in parenchymal liver cells might be so interpreted whereas uptake by Kupffer cells would suggest that the mucoprotein-like material (intrinsic factor) was simply being treated by the reticuloendothelial system as foreign to the blood stream.

Despite intensive effort in many laboratories during the past decade intrinsic factor has not yet been isolated in pure form. It is presently believed to be a mucoprotein or a mucopolypeptide (3, 23, 24). Even this is uncertain, however. Glass has speculated that intrinsic factor may, in fact, be a prosthetic group which can attach itself to many substances present in normal gastric juice (25). Even the exact site of secretion of intrinsic factor in the stomach is unknown. Autoradiographic studies suggest the pepsinogen-elaborating cells in the rat (26), but patients have been found who secrete pepsin but not intrinsic factor (27).

Present knowledge (6) suggests that the vitamin B_{12} of ingested animal protein is released from loose binding to this protein by an acid environment as it passes through the stomach. Intrinsic factor then binds this freed vitamin B_{12}. This bond will occur in an acid medium and appears to be considerably stronger than that of vitamin B_{12} to animal protein generally. The vitamin B_{12}-intrinsic factor complex then appears to be adsorbed to the intestinal mucosa. Present evidence indicates that this adsorption is not of vitamin B_{12} to the intestinal mucosa as previously suggested (28) but rather that intrinsic factor laden with vitamin B_{12} unites with an intestinal receptor or acceptor (29, 30) through a calcium-dependent bond (6, 24, 29). Within the past year evidence was presented (6) for the next step in this sequence of events. It was found that *in vitro* an aqueous extract of rat intestine was capable of releasing vitamin B_{12} from its bond to rat intrinsic factor much more readily than from its bond to human intrinsic factor. Moreover, the calcium-dependent bonding of rat intrinsic factor to rat intestinal wall more quickly became insusceptible to calcium-chelating ethylenediaminetetraacetate (EDTA) than when human intrinsic factor carried the vitamin B_{12}. This led to the hypothesis that at or within the intestinal mucosa there occurs a species-dependent release of vitamin B_{12} from intrinsic factor.

However, the possibility existed that the more ready release of vitamin B_{12} from rat intrinsic factor might be an artefact due merely to a weaker or more susceptible binding of vitamin B_{12} by rat intrinsic factor than by human intrinsic factor, which indeed appears to be more resistant to destruction by various means. To overcome this argument it would be necessary to demonstrate that human intestinal extract would release vitamin B_{12} more readily from human intrinsic factor than from rat intrinsic factor. This experiment was undertaken some months ago. The methods used for preparation of stomach homogenates (31) and intestinal extract (6) have been previously recorded. The source of human ileal extract was a piece of normal mid-ileum removed during operative revision of an ileostomy. Table 1 demonstrates the results of this experiment. As you see, not only is rat intestinal extract capable of releasing vitamin B_{12} preferentially from rat intrinsic factor, but human intestinal extract is capable of releasing vitamin B_{12} preferentially from human intrinsic factor. It has not yet been possible to extend this experiment to the hog because we have not yet been able to prepare a hog intestinal extract free of intrinsic factor (32). Thus it would appear that the species-dependency of intrinsic factor plays a role neither in the binding of vitamin B_{12} to intrinsic factor or in the attachment of the intrinsic factor-vitamin B_{12} complex to the intestinal mucosa, but rather in the next stage: the release of vitamin B_{12} from intrinsic factor by a presumed enzyme in the ileal mucosa. It is possible that this is the stage at which the mechanism producing refractoriness to intrinsic factor (33) is operative. Species specificity of 'release' of vitamin B_{12} from intrinsic factor provides a simple explanation for the more rapid intestinal absorption of vitamin B_{12} attached to 'homologous' intrinsic factor (6, 30).

TABLE 1. *Species Specificity of Intestine Extract-Mediated Release of Vitamin B_{12} Bound to Intrinsic Factor*

Species of Intrinsic Factor Bound to Vitamin B_{12}	Added Intestine Extract	Vitamin B_{12} Made Dialyzable, %
Human (stomach homogenate)*	None	0.8
	Human†	30.4
	Rat	2.6
Rat (stomach homogenate)	None	3.0
	Human†	1.6
	Rat	38.2

* Prepared from fresh stomach obtained during subtotal gastrectomy for duodenal ulcer. †Prepared from normal mid-ileum obtained during operative revision of an ileostomy. Human intestine extracts were inactive when prepared from autopsy material or from the distal 8 inches of fresh terminal ileum, obtained during resection for carcinoma of ascending colon.

Specific malabsorption for vitamin B_{12} has recently been described in adults (34) and in children (35–38). The recent experimental studies here reviewed would suggest that this category in the etiologic classification of the megaloblastic anemias may now be subdivided into at least two groups: that due to reduction or absence of the small intestinal receptors for intrinsic factor, and that due to reduction or absence of the 'releasing factor' in the small intestine.

In summary, the absorption of physiologic amounts of vitamin B_{12} appears to take place in the ileum. This process is dependent on an adequate secretion by the gastric mucosa of an 'intrinsic factor' of as yet unknown chemical structure which binds vitamin B_{12}, and then attaches itself to the intestinal mucosa in the ileum by a calcium-dependent bond. Vitamin B_{12} is then released either at or within the intestinal mucosa by a substance of unknown but presumably enzymatic nature which has a preferential species-dependent action upon the intrinsic factor-vitamin B_{12} bond.

The next step in dissection of the sequence of events in the absorption of vitamin B_{12} will be to determine whether the release of vitamin B_{12} from intrinsic factor takes place 'at' or 'within' the intestinal mucosa, and then to follow the path the vitamin takes within the intestinal mucosal cells and thence into the blood stream. Among the many questions not yet answered are those

relating to the chemical nature of intrinsic factor, the mechanism of release of vitamin B_{12} from intrinsic factor, the possibility that intrinsic factor itself may pass into the intestinal mucosal cells or even beyond them into the blood stream, and the possible existence of one or more similar or identical steps in the absorption of other nutrients. Thus, although Austin Flint correctly surmised the importance of the lack of gastric secretion one hundred years ago, there are still things that one may do in the laboratory and at the bedside in the further pursuit of his correct hypothesis concerning the cause of pernicious anemia.

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Comparative enzyme apparatus of the gut mucosa[1]

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The small intestine is a functionally specialized organ, and it is in keeping with modern biochemical knowledge to expect that it has an equally specialized metabolic apparatus. Only recently has our information about both synthetic and degradative enzyme systems become sufficiently complete so that the attempt could be made to characterize the enzymic equipment of an organ for comparison with its physiological function. The intestine has not been a favorite organ of study by biochemists, but even so, a considerable body of information is available. We have collected the scattered studies and some scraps of information which bear on

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the metabolism of the small intestine; these form the basis of this introduction to a metabolic appraisal of the intestinal tract.

Our purpose is to describe in broad outline the nature of the major metabolic systems present in the adult small intestine. We will consider it mainly as a functional system, in terms of its enzymes rather than its chemical anatomy. For comparison we will consider the distribution of the same enzymes in other tissues or in the same species at different ages. It must be recognized that few of the data were collected for the purpose in which we are using them, so detailed conclusions about the metabolism of the intestine are not possible. Yet the data collected here show the elaborate and active metabolism of this tissue and some of its major characteristics. Systematic studies can expand this picture of an organ in the same way that the metabolism of a particular species of organism can be described in relation to its known life habits and in relation to other similar organisms.

The unique tissue of the small intestine is the mucosa. It makes up perhaps one-fifth of the mass. The rest consists largely of connective tissue and smooth muscle, not demonstrably different from similar tissues elsewhere in the body. We have purposely ignored the description of the extracellular secretions of this mucosa since there is an adequate literature on the subject. The active intracellular life of the mucosa is our real concern. The mucosa is rapidly renewed, with half the cells turned over every 2 or 3 days. The mucosal cell replacement is as rapid as that occurring in the hematopoietic system. This is one reason why factors such as irradiation or folic acid antagonists which interrupt cell reproduction primarily effect both the gastrointestinal tract and the bone marrow. There are some 8 liters of fluid with its contained electrolytes and proteins secreted daily by the intestine and its associated glands which must be absorbed. This fluid comprises an extracellular compartment of some importance. In addition to formation and reabsorption of this fluid, the intestine must also absorb the digested products of the diet, many of them by active energy-requiring processes. In regard to the work of active absorption: if the energy required to absorb an amino acid is the same as to activate it for protein synthesis, say one phosphate bond, it is apparent that the intestine does as much work in absorbing amino acids as does the rest of the body in making them into proteins. The same considerations will hold for other substances actively transported by the intestine. It is apparent then, that cell growth and energy utilization for synthesis and work are the major needs to be supplied by intestinal metabolism.

CARBOHYDRATES AND ENERGY PRODUCTION

The large energy demands of the intestine are apparently supplied from carbohydrate with very little coming from the metabolism of fat and amino acids. The pathways of this carbohydrate metabolism are the familiar ones. The immediate product of hexokinase action, glucose-6-phosphate, is shown in the central role in figure 1. Low levels of glycogen occur (5), and are formed both by the familiar phosphorylase and by the more recently discovered UDP-glucose route. Both glycolysis and oxidation of hexosemonophosphate are significant reactions in the intestine. Pyruvate derived from these reactions can be completely oxidized via the citric acid cycle. Glycolysis is perhaps less used than hexosemonophosphate oxidation. The ratio of carbon dioxide derived from carbon six to carbon one of glucose is reported to be only 0.17 (15) in comparison with the ratio of 1.0 in liver (16); this indicates that a large part of the glucose is metabolized through the hexosemonophosphate pathway, instead of by glycolysis. Two enzymes of the hexosemonophosphate shunt have already been identified in the gut wall (17). Even so, glycolysis is an exceedingly active process in the intestine as shown in table 1. Aerobic glycolysis is as active as that seen in many tumor tissues. The depression of glycolysis by oxygen (Pasteur effect) is minimal in the upper portions of the intestinal tract, but increases toward the lower end (table 2). This, however, as well as lactic acid production (13) may show species variation (12). Such variations in metabolic reactions along the length of the intestine are not uncommon.

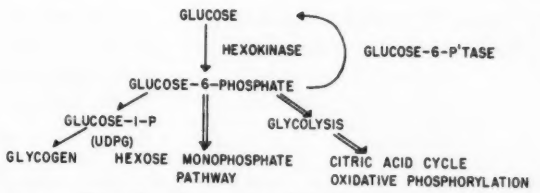

FIG. 1. General pathways of carbohydrate metabolism by the intestine. References: hexokinase (1, 2); glucose-6-phosphatase (3); glycogen synthesis (4–6); fructose metabolism (8, 9, 11); miscellaneous (7, 10, 12–14).

The intestine is well supplied with both the enzymes of the citric acid cycle and citric acid itself (table 3) (20, 24–31, 33). There is an adequate complement of the cytochromes and cytochrome oxidase for terminal oxidation as illustrated in table 4. Oxidative phosphorylation, the prime source of chemical energy for this highly aerobic tissue, is as efficient as it is in liver (table 5). Uncoupling of phosphorylation from oxidation as by dinitrophenol (34) results in diminution of a number of intestinal processes, such as carbohydrate absorption. The relative activity of specific enzymes in the intestine, compared with other tissues, is known for a number of enzymes concerned with the metabolism of carbohydrate. A few are illustrated in figure 2. The relatively high activity of lactic dehydrogenase present in the intestine is consistent with the large combustion of carbohydrates for energy as well as with the generation of reduced pyridine nucleotide for synthetic purposes. The chemical energy formed in the intestine appears to be stored only in the muscle as creatine phosphate,

TABLE 1. *Representative Values of Oxygen Uptake and Glycolysis in Hamster and Rat Intestine*

	Q_{O_2}	Lactic Acid	Anaerobic CO_2
	μl/mg dry wt/hr.		
Hamster*			
duodenum	22.7	9.7	11.1
jejunum	14.3	10.6	14.7
ileum	10.0	3.3	10.4
Rat†			
duodenum	8.8	11.1	10.4
jejunum	15.6	16.5	16.4
ileum	5.3	5.5	4.0

Values from (13, 18). *0.2% glucose in medium. †0.3 % glucose in medium.

TABLE 2. *Extent of the Pasteur Effect (Depression of Glycolysis by Aerobic Conditions) Shown by Intestinal Segments*

Species	Duodenum	Jejunum	Ileum	Colon	References
Rabbit	0.1				(20)
Rat (mucosa)	0.6	0.9	0.9	0.3	(18)
Rat	0.9	1.2	0.3		(13)
Hamster	0.9	0.7	0.3		(13)

Values are ratio: aerobic glycolysis/anaerobic glycolysis.

TABLE 3. *Citric Acid Content of Rat Tissues (mg/100 gm)*

References	Small Intestine	Kidney	Heart	Liver
(21)	7.6	3.8	5.3	1.2
(22)		2.4		2.3
(23)	7.8	5.1	3.8	

and must exist in the mucosa largely in the form of the adenosine phosphates.

NUCLEIC ACIDS

The rapid rate of cell replacement in the intestinal mucosa, and the synthesis of enzymes for secretion, indicates that this tissue must have a massive synthetic metabolism for nucleic acids and proteins. Yet surprisingly little has been done on the component enzymes for these reactions. It is known that DNA phosphorus turns over at least as rapidly as the cells (38). A number of the steps of purine and pyrimidine synthesis are known to occur in the mucosal cells (table 6). The ribonuclease which degrades RNA is very active in the intestinal mucosa as would be expected (45) and in the mouse it is more active in intestine than in kidney and liver. Thymidine phorphorylase is also very active in the intestinal mucosa (51).

AMINO ACIDS

Few direct studies of protein synthesis have been made on the intestinal mucosa. Apparently none have dealt directly with amino acid activating enzymes (67, 196). Nevertheless, the intestine is one of the sites of highest activity concentration of parenterally administered radioactive amino acids (57, 68–70). The concentrating ability decreases with age and in vitamin B_6 deficiency (71). The intestine holds, apparently in free form, a generous pool of amino acids (table 7) and derivatives (table 8). Extreme rapidity of protein synthesis in the intestinal mucosa can be dramatically shown when the oral feeding of iron acts as a stimulus for ferritin synthesis (267). Shortly afterward, cadmium induces the crystallization of ferritin from crushed mucosal preparations. A summary of data on protein renewal by the gut is given in table 9.

The active degradation of the amino acids by a variety of routes which is so characteristic of the liver and which occurs to a smaller extent in the kidney is minimal in the intestine. A number of the amino acids can be transaminated in the intestinal mucosa, although the variety and activity of these enzymes is not impressive. The capability does not suggest that transamination reactions are involved directly in the active transport of amino acids by the mucosa (table 10). Interesting too are the variety of amino acid decarboxylations catalyzed by the intestine (table 11). Though not quantitatively large, these reactions frequently result in pharmacodynamically active amines with considerable local and systemic functional effects. It should be noted that at least certain of these reactions are confined to particular cell types within the intestinal mucosa or arranged along the intestinal tract. It is of interest that both of the systems for inactivating histamine, diamine oxidase (109) and histamine methylase (119), are present in the intestine. The relative level of the latter is high (119). A number of other reactions involving amino acids are performed by the intestine (tables 12 and 13); while these are presented for the sake of completeness, they are quantitatively minor.

The one amino acid richly supplied with metabolic routes in the intestine is glutamic acid, possibly because of its close relation to the energy-yielding reactions of carbohydrate metabolism, but also possibly for reasons related to the functional activity of the intestine (at least glutamine present in gliadin is thought to play a role in the genesis of certain types of malabsorption

TABLE 4. *Complements of Cytochrome Oxidase and Cytochrome C*

	Intestine	Kidney	Heart	Liver	References
	Cytochrome Oxidase ($mm^3O_2/mg/hr$)				
Rat	0.22	1.13	1.64	1.06	(39)
Rat	0.98*	2.49	2.44	1.51	(35)
Man	0.50	3.50	8.20	2.80	(36)
	Cytochrome C (μg/gm)				
Man	9	24	102	23	(36)
Beef	6†	29	191	14	(37)

* Large intestine. †Stomach.

TABLE 5. *Oxidative Phosphorylation by the Intestine*

Substrate	Intestine	Liver
Citrate 0.01 M	2.9	2.4
Succinate 0.033 M	2.3	1.9

Values are P:O ratio (μmoles P esterified/μatom O_2 used). From (32).

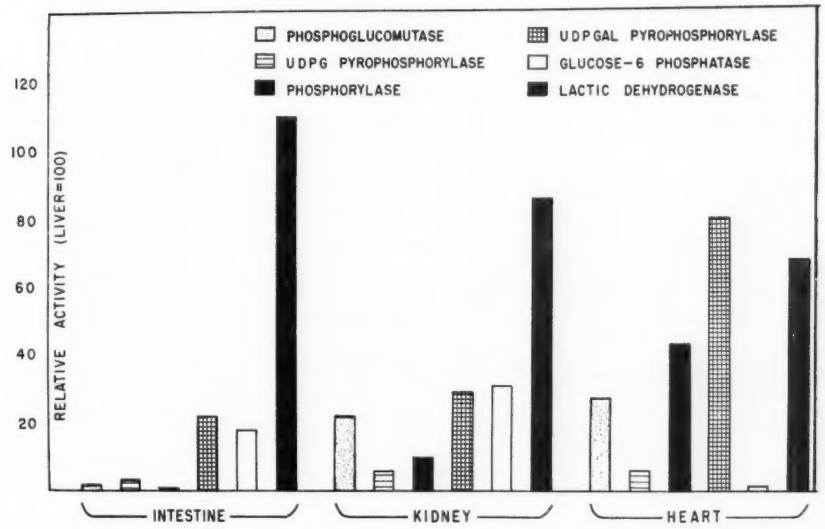

FIG. 2. Activity of 6 enzymes, involved in carbohydrate metabolism, in various tissues (all data from the rat except for mouse lactic dehydrogenase). In each case the liver value was assumed to be 100. From data in (3, 5, 7, 14).

TABLE 6. *Enzymes Involved in the Metabolism of Nucleic Acids and Precursors Identified in the Intestine*

Enzyme or Reaction	Species	References
Ribonuclease, acid & alkaline	mouse, rat	(45)
Deoxyribonuclease	mouse	(46)
5-Nucleotidase	rat, rabbit, dog, ox, calf	(47, 48)
5-Adenylate deaminase	mouse, rabbit	(48, 49)
Adenosine deaminase	rabbit, calf	(48, 49, 52)
Thymidine phosphorylase	rabbit	(51)
Guanase	mouse	(48)
Xanthine oxidase	man, ox, dog, cat, rabbit, hedgehog, chicken, mouse	(53–55)
System for: adenosine triphosphate → inosine, inosine-monophosphate, xanthine	sheep	(56)
Utilization of 4-amino-5-imidazole carboxamide	rat	(43)
Formation of histones of nucleoprotein	rat	(57)
Nucleic acid turnover and content		(40–42, 44, 58, 180)

TABLE 7. *Intestinal Content of Free Amino Acids and Peptides*

Compound	Species and Location	Concentration *μmoles/ml water*	Ratio: intestinal conc. / liver conc.	References
Carnosine	Ox (muscularis)	1.1		(76)
Glutamine	Sheep (mucosa)	1.5	0.7	(72)
	Cat (mucosa)	4.5	1.0	(72)
	Dog (whole)	4.6	1.2	(73)
Glutamic acid	Sheep (mucosa)	6.8	0.9	(72)
	Cat (mucosa)	6.3	1.9	(72)
Glycine	Mouse (whole)	5.1	1.1	(74)
Glutathione	Rabbit (whole)	3.1	0.4	(75)

(124)). The major reactions of glutamine and glutamic acid in the intestine are shown in figure 3. These are the familiar reactions occurring in other tissues. The importance of the various pathways may be inferred from the relative activity of these enzymes shown in table 14. Glutamine transamidinase (glutaminase II) is one of the more important enzymes.

Several of the steps of the urea cycle occur in the intestinal mucosa. Many species are known to possess arginase and so can form urea, at least from preformed arginine, and perhaps can synthesize arginine to some extent (table 15).

LIPIDS

As mentioned earlier, lipid degradation as a source of energy is of minor importance in the intestinal mucosa. The intestine, however, contains large amounts of many lipids (table 16). It can carry out elaborate storage and syntheses of fatty acids (158, 165–171, 173, 175, 176, 184), vitamin A from carotene (172, 177), cholesterol (59, 156, 158, 174, 178, 179), (the gut possesses a cholesterol esterase 181–183), phospholipids (162, 185–188, 191–193, 195, 197), choline derivatives (198–202), and glycerides. Intestinal synthesis of glycerides (203, 209) does not depend upon glycerol as the immediate precursor (204, 205). The enzyme glycerol kinase (glycerol → L-α-glycerophosphate) cannot be demonstrated (205). However, the intestine does contain L-α-glycerophosphate (189, 190). Exogenous L-α-glycerophosphate added to intestinal homogenates becomes incorporated into glycerides and glycerol (205). The likely origin of L-α-glycerophosphate for these reactions is from the glycolytic pathway (206), possibly by fructose cleavage (8, 205), through dihydroxyacetone phosphate with the mediation of L-α-glycerophosphate de-

TABLE 8. *Intestinal Content of Amino Acid Derivatives*

Compound	Species and Intestinal Location	Concentration *μmoles/ml water*	Comment	References
Creatine	Rat (muscularis)	6.4	⅓ of conc. in heart	(79)
Ergothioneine	Rat (whole)	0.033	Liver conc. 22 times greater	(80)
Histamine	Rat (whole)	0.18	Over 3 times lung conc.	(81, 85)
	Rabbit (whole)	0.06	Greater than lung conc.	(81)
Serotonin	Chick (whole)	0.037		(84)
	Rabbit (whole)	0.120		(81)
	Rat (whole)	0.028	10 times brain conc.	(81)
Phosphoethanolamine	Rat (whole)	0.53	Higher conc. in Peyer's patches and colon	(82)
Taurine	Rat (whole)	14.3	Diff. between males and females	(83)
	Guinea pig (whole)	3.9	Rat liver contains taurine; none in guinea pig or rabbit liver	(83)
	Rabbit (whole)	4.5		(83)
Guanidoethylseryl phosphoric acid diester	Lubricus terrestris	occurs	An arginine analogue	(77)
Sarcosine	Autolysates starfish caeca	occurs	N-methylglycine	(78)

TABLE 9. *Summary of Various Data on Protein Content and Renewal Rate by the Intestine**

Event	Magnitude	References
Protein turnover to replace lost cells	7 gm/day	(38)
Protein turnover to provide for digestive enzyme synthesis	10 gm/day	(59)
Collagen content of intestine	12 gm/100 gm	(60)
Soluble collagen precursors not demonstrable in human small intestine		(61)
Paneth cells the likely site of digestive enzyme synthesis		(65, 66)
Protein content of gut increases during pregnancy and lactation		(62)
Intestine a site of protein catabolism and storage		(63, 64)

* Based on figures in the references.

hydrogenase and reduced diphosphopyridine nucleotide (207, 208).

glucose →
fructose → dihydroxyacetone phosphate → L-α-glycerophosphate → glycerides
others? →

VITAMINS AND COFACTORS

A survey of the intestinal mucosal content of the various vitamins and coenzymes reveals a remarkably even pattern (table 17). The concentrations of most of these substances are significant, but lower than those present in the kidney and liver. If analyses were available for the intestinal mucosa alone, the relative content of this tissue would compare favorably with the other organs for most of the compounds. A few exceptions to this type of distribution are noteworthy. There is practically no vitamin A in the intestine, while there are relatively high amounts of ascorbic acid. The content of vitamin D, surprisingly enough, is lower than in many other tissues. Most of the vitamins, however, are present in relative amounts similar to those shown for thiamine and pyridoxine. It should be emphasized that this distribution of pyridoxine also does not lend particular support to a special function for this compound in the active absorption of amino acids. However, since a similar type of active transport of amino acids occurs in all cells of the body, a unique concentration of pyridoxine in the gut mucosa would not be expected. The intestine not only absorbs vitamins but is also involved in their metabolism (such as phosphorylating riboflavin (236) and converting carotene to vitamin A (172, 177)).

ENZYME ADAPTATION

In addition to the elaborate and active complement of enzymes which the intestine shows and which must be maintained in a dynamic state to accomplish the work of the organ and regeneration of cells, this tissue shares with other tissues of the body a certain plasticity of its enzyme pattern. When the physiological state of the animal is altered in almost any way, the activity and apparently the amount of particular enzymes in the intestinal mucosa changes. The specific adaptability of digestive enzymes in the alimentary tract first observed by Pavlov has been reviewed by Shlygin (258). The intracellular enzymes also change. In table 18 are a variety of procedures which alter the physiological state of the animal and affect particular enzymes of the intestine. Some of these procedures, such as the treatment of the chick embryo with phenylphosphate to increase the gut alkaline phosphatase activity, appear to be substrate inductions. Others are changes of the adaptation type in the larger sense which occur in animals. Enzyme changes are produced by substrates and products of the enzymes as well as by hormones. It is obvious, but still unrealized, that the correlation of these induced enzyme changes in the gut with changes in the functional activity of the gut occurring at the same time offers a new

TABLE 10. *Summary of Several Known Examples of Intestinal Transamination and Deamination of Amino Acids*

Amino Acid		Reaction	Species	References
Glutamine	→	glutamic acid (in presence of phosphate; glutaminase I)	Guinea pig, rat	(86, 87)
	→	glutamic acid (in presence of keto acid; glutaminase II)	Guinea pig	(86)
Asparagine	→	aspartic acid (asparaginase)	Guinea pig	(88)
Glutamic acid	→	alanine (glutamic-pyruvate transaminase)	Rat, dog, cat, rabbit	(89, 92, 93)
Glutamic acid	→	aspartic acid (glutamic-oxaloacetic transaminase)	Rat, dog	(89, 91)
Leucine		Formed from keto acid	Rat, cat	(90)
Valine		Formed from keto acid	Rat, cat	(90)
Methionine		Perhaps formed from keto acid	Rat	(90)
Aspartic acid		No L-aspartic oxidase	Rat	(94)
D-forms		If D-amino acid oxidase present, activity is minimal		(95)

TABLE 11. *Data on Intestinal Decarboxylation of Amino Acids*

Amino Acid	End Product	Species and Comment	References
Histidine	Histamine	Man, pig, rat, guinea pig	(96–99)
Dihydroxyphenylalanine	Hydroxytyramine	Guinea pig	(97)
5 - Hydroxytryptophan	5 - Hydroxytryptamine	Mouse, rat, rabbit, chick, guinea pig. Enzyme inhibited by vitamin B_6 deficiency and by α-methyldopa.	(81, 84, 100)
Glutamic acid	γ-Aminobutyric acid	Rat	(90)
Alanine	Ethylamine	Rat	(90)
Leucine	Isoamylamine	Rat	(90)
Valine	Isobutylamine (? Minimal)	Rat	(90)

TABLE 12. *Sulfur Amino Acids and Sulfur Compounds in the Intestine*

Reaction or Product	References
Identification in wall of cystathionine, cystine, methionine, glutathione, taurine, ergothioneine	(58, 75, 80, 83, 108, 115)
Uptake of injected methionine, cystine, taurine, methionine sulfoximine	(101–105)
Methionine:	
Formation from keto acid	(90)
Cystine formation (probable)	(107)
Production of sulfoxide	(122)
Methyl transfer to catechol-o-methyl transferase and histamine-methylating enzyme	(118–121)
Inhibition of amino acid absorption, alteration of alkaline phosphatase level	(116, 117)
Glutathione synthesis	(106)
Interconversion of formate with sulfur amino acids	(58)
Cysteine desulfhydrase	(123)
Adenosine-3′-5′-phosphosulfate	(56, 110, 112, 113)
Phenol sulfokinases	(114)

approach to identify the enzymic bases of functional activity of the intestine. The amount of various substances within the intestinal wall can also be varied (table 19), perhaps due in part to alteration of the enzymic makeup by different stimuli.

TABLE 13. *Some Additional Reactions of Amino Acids in the Gut Wall*

	References
Tyrosine-Phenylalanine:	
phosphotyrosine hydrolyzed	(128)
minimal homogentisic acid oxidase	(129)
presence of tyrosine iodinase	(130)
formation of iodoprotein	(131)
accumulation of radioactive tyrosine	(70)
Histidine:	
lack of significant histidine breakdown	(134)
splitting of 2 histidine derivatives (carnosine, anserine)	(155)
Tryptophan:	
L-acetyltryptophan absorbed, not cleaved	(90, 135)
lack of significant indolylpyruvic acid tautomerase	(136)
Others:	
possible demethylation of sarcosine to glycine	(137)
conversion of glycine to serine	(138)
presence of a dehydro peptidase	(139)
acylase active against some amino acyl compounds	(141, 142)

TABLE 14. *Activity of Some Intestinal Enzymes Involved in Glutamic Acid Metabolism*

	Activities (Liver = 100)
Glutamic Dehydrogenase, rat	7
Glutamic-Pyruvate Transaminase, rat	9
Glutamine Synthetase, guinea pig	40
Glutaminase I (phosphate), rat	60
Glutaminase II (α-keto acid), guinea pig	100

References: (73, 86, 87, 89, 93, 125, 132, 133, 127).

TABLE 15. *Intestinal Handling of Urea*

Event	References
Urea splitting enzyme due to bacteria	(145)
During uremia, electrolytes (and possibly urea) accumulate in gut wall	(146)
Urea passes from alimentary mucosa to lumen	(147, 148)
Arginase (arginine → urea + ornithine) in gut of ox, rabbit, mouse, possibly dog (not in rat, cat, pigeon)	(149, 150–152, 154)
Intestinal arginase has broad specificity	(150, 154)
Additional enzymes of urea cycle (carbamyl aspartic acid synthetase, ornithine transcarbamylase) may be present in gut wall	(143, 144, 153)

XUM

TABLE 16. *Tissue Content of Various Lipids*

Component	Species	Units	Intestine	Kidney	Heart	Liver	References
Cholesterol							
(total)	Guinea pig	mg/100 gm	278	316		252	(156)
(esterfied)	Guinea pig	mg/100 gm	37	47		40	(156)
(total)	Dog	mg/100 gm	228			103	(158)
(total)	Rat	mg/100 gm	170			170	(159)
(total)	Mouse	mg/100 gm	221	442		514	(163)
Total 'Lipids'	Rat	mg/gm	78.3	38.0	35.0	42.9	(162)
Methostenol	Rat	gm/100 gm lipid	1.5				(165)
'Phospholipid'	Rat	*P*, as % of total lipid	0.43	2.39	2.5	2.43	(162)
Fatty Acids	Rat	mg/gm	35.7			26.1	(159)
	Rat	total mg	110			211	(164)
Sphingosine	Dog	μm/gm dry lipid-free residue	23.1	34.6	18.0	9.3	(161)
Inositol	Dog	μm/gm dry residue	9.6	13.2	7.4	13.0	(157)
Phosphoethanolamine	Rat	mg/100 gm	6	Trace	20	17	(82)
Sterol	Rat	mg/gm	2.43	4.29		2.43	(160)
Unsaponifiable matter	Rat	gm/100 gm	0.370	0.617		0.403	(160)

TABLE 17. *Tissue Content of Vitamins and Other Cofactors*

Substance	Species	Units	Small Intestine	Kidney	Heart	Liver	References
Pantothenic Acid	Rat	Liver = 100/gm dry wt.	17[a]	51	54	100	(232)
Coenzyme A	Rat	arbitrary/gm	13		32	91	(221)
	Rat	arbitrary/gm	26	74	4[c]	132	(210)
Glutathione	Rabbit	mg/gm	0.78			2.04	(75)
	Rat	mg/gm		1.53	0.19	2.74	(212)
	Guinea pig	mg/gm	1.7		0.45[b]	3.45	(218)
	Guinea pig	mg/gm	2.07	1.72		3.51	(222)
Ubiquinone	Rat	μg/gm	45	91		137	(160)
Ascorbic Acid	Rat	mg/gm	0.48	0.25		0.29	(220)
	Rat	mg/gm	0.20	0.18	0.08	0.25	(213)
	Cow	mg/gm	0.22				(214)
	Guinea pig	mg/gm	0.13	0.15		0.17	(222)
Vit. B_{12}	Man	μg/gm	0.024[c]	0.103		0.614	(215)
	Dog	μg/organ	2.47	3.28	3.55	20.06	(223)
Vit. D (after 200,000 unit dose)	Dog	I.U./100 gm	1.44	8.88	5.73	0.82	(216)
Iron	Rat	mg/100 gm	1.6	4.3	7.2	6.1	(217)
Copper	Man	mg/100 gm	2	5	3	8	(36)
Folic Acid	Guinea pig	μg/gm	48			1531	(219)
	Rat	μg/gm	384			3051	(219)
Folinic Acid	Guinea pig	μg/gm	8			181	(219)
	Rat	μg/gm	161			890	(219)
Vit. A	Rat	I.U./gm	0	8.3		61	(160)
3′,5′-Diphosphoadenosine	Rabbit	mμm/gm wet wt.	0	3.0	0	18.0	(112)
Riboflavin (and coenzymes)	Rat	μg/gm	5.15			29.8	(224)
	Beef	μg/gm	9.14		2.23[d]	31.50	(230) (231)
	Beef	μg/gm	11.45[c]				(230)
Adenosine triphosphate	Rat	μm/gm	1.45	1.8[e]	6.2[d]	1.5	(225) (226) (227) (229)
Adenosine diphosphate	Rat	μm/gm	0.77		1.05[d]	13.1	(225) (226) (227)
Adenosine monophosphate	Rat	μm/gm	0.19		0[d]	0.5	(225) (226) (227)
Diphosphopyridine nucleotide	Rat	μm/gm	0.26	0.66	0.73	0.87	(225) (228)
Creatine phosphate	Rat	μm/gm	1.94[e]	0.11	2.84	0.05	(229) (232)
Inositol	Rat	mg/gm dry tissue	1.70[a]	8.80	1.10	2.10	(233)
	Dog	mg/gm dry tissue	1.79	2.46	1.38	2.42	(157)
Biotin	Man	μg/gm wet tissue	0.06[f]	0.32	0.12[b]	0.65	(234)
	Rat	μg/gm dry tissue	0.58[a]	0.96	0.17	0.88	(233)
Pyridoxine	Man	μg/gm wet tissue	0.5[f]	3.5	3.7[d]	7.8	(234)
	Rat	μg/gm wet tissue	1.50[g]	4.16	4.62	5.28	(235)
	Rat	μg/gm dry tissue	1.30[a]	3.60	3.30	2.50	(233)
Nicotinic Acid	Rat	μg/gm dry tissue	190[a]	530	520	530	(233)
Thiamine	Rat	μg/gm dry tissue	9.7	38	11	31	(233)

[a] Intestinal tract. [b] Muscle. [c] Mucosa. [d] Skeletal muscle. [e] Taenia. [f] Bowel. [g] Stomach.

TABLE 18. *Effect of Various Procedures on Intestinal Enzymes*

Stimulus	Change*	Species	References
Alkaline phosphatase			
Rickets	None	Dog	(257)
Desoxycorticosterone	Inc.	Rat	(248)
Adrenalectomy	None	Rat	(237)
Adrenalectomy	Dec.	Rat	(245)
Colchicine	Dec.	Rat	(255)
Castration (males)	None	Rat	(238)
Disodium phenylphosphate	Inc.	Chick (embryo)	(251)
Vitamin C deficiency	None	Guinea pig	(254)
Zinc deficiency	Dec.	Chick	(240)
Vitamin D	Inc.	Rat	(256)
Growth	Inc.	Guinea pig	(244)
Thyroxin	Inc.	Rat	(246)
Fasting	Dec.	Rat	(252)
Feeding: mixed diet	Inc.	Rat	(239)
phosphoprotein	Inc.	Rat	(250)
methionine	Inc.	Rat	(116)
tryptophan	Dec.	Rat	(116)
Acid phosphatase			
Adrenalectomy	None	Rat	(237)
Castration (males)	None	Rat	(238)
Disodium phenylphosphate	None	Chick (embryo)	(251)
Xanthine oxidase			
Molybdenum deficiency	Dec.	Rat	(241)
Iron deficiency	Dec.	Rat	(241)
Riboflavin deficiency	Dec.	Rat	(224)
Histaminase			
Adrenalectomy	Dec.	Cat	(242)
Hypophysectomy	Dec.	Cat	(242)
Histamine	None	Rat	(259)
Cholinesterase			
Thiamin deficiency	Inc.	Pigeon	(243)
Sucrase			
Hyperthyroidism	Inc.	Dog	(247)
Age (?)	Inc.	Pig	(50)
Adenosinetriphosphatase			
Adrenalectomy	Dec.	Rat	(1)
Hexokinase			
Adrenalectomy	None	Rat	(1)
Low fat diet	Inc.	Rat	(249)
Amylase (intestinal)			
Starvation	Dec.	Rat	(261)
Phytase			
Vitamin D	Inc.	Rat, chick	(21, 253)
Glutamic transaminases (pyruvic, oxaloacetic)			
Starvation	None	Rat	(89)
Cortisone	Inc.	Rat	(89)
Adrenalectomy	Dec.	Rat	(89)
Arginase			
Castration	None	Mouse	(260)
System for converting carotene to vit. A			
Thyroidectomy	Dec.	Rat	(268)
Diabetes mellitus	Dec.	Rat	(266)
System for conjugating o-aminophenol with glucuronide			
Cortisone	Dec.	Rat	(262)

* Inc. = increase. Dec. = decrease.

TABLE 19. *Alteration of a Number of Substances Within the Intestinal Wall*

Substance Within Intestine	Stimulus	Change*	Species	References
Glutathione	Scurvy	None	Guinea pig	(222)
Serotonin	Vit. B_6 deficiency	Dec.	Chick	(84)
Ascorbic acid	Scurvy	Dec.	Guinea pig	(222)
	Insulin	None	Rat	(220)
	Methylene chloride	Inc.	Rat	(211)
Dehydroascorbic acid	Scurvy	Inc.	Guinea pig	(222)
Histamine	Adrenalectomy	Inc.	Rat	(194)
	Adrenalectomy	None	Cat	(242)
	Hypophysectomy	None	Cat	(242)
	Starvation	None	Cat	(263)
Iron (total)	Hemochromatosis	None	Man	(264)
	Ingestive siderosis	Inc.	Man	(264)
	Deficiency diet	Inc.	Rat	(265)
Citrate	Vit. D	Inc.	Rat	(21)
	Cortisol	None	Rat	(23)
	Fluoroacetate	Inc.	Rat	(23)
Coenzyme A	Thyroidectomy	Dec.	Rat	(221)
Apoferritin (or ferritin)	Iron	Inc.	Guinea pig, horse	(111, 267)
Fatty acids	Diabetes	Dec.	Rat	(159)
Cholesterol	Bile acids	None	Mouse	(163)
	Diabetes	None	Rat	(159)
	Scurvy	Inc.	Guinea pig	(156)
	Scurvy + insulin	None	Guinea pig	(174)
Secretin	Hypophysectomy	Dec.	Rat	(32)
Folinic acid	Aminopterin	Dec.	Rat	(219)
	Aminopterin	None	Guinea pig	(219)

* Dec. = decrease. Inc. = increase.

SUMMARY

This cursory analysis of the enzymes of the small intestine indicates that it is a tissue with an unexpectedly high metabolic activity whose energy production largely comes from carbohydrate by familiar routes. One part of this energy is utilized for the very rapid cell growth and replacement with synthesis of proteins, nucleic acids, structural lipids and other substances necessary for this tissue. Opposed to this intense synthetic activity, there is little degradation of the substances below the level of the simple building blocks for the more complex compounds. Exceptions to this are the elaborate metabolism of glutamine and glutamic acid, as well as certain other amino acids in small amounts to pharmacologically active amines. The hydrolytic activities related to the secreted enzymes of digestion have apparently been of little hindrance in the mucosal preparations studied. The systems which have been discussed include no unique reactions or cofactors that could be implicated in any way with the active absorption which is such a prominent feature of the function of the intestine. A large but indeterminate part of the energy provided from carbohydrate metabolism in the intestinal mucosa must be used for this active transport of materials across the gut wall.

The pattern of enzymic distribution in the intestinal mucosa appears to change along the length of the intestine and also to change from time to time in relation to the altered state of the whole animal (adaptation). A

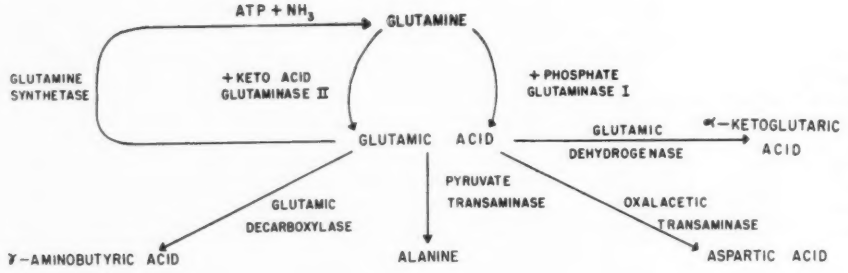

FIG. 3. Major metabolic pathways of glutamic acid in the intestine. References: glutaminases I and II (86, 87); glutamine synthetase (86); glutamic decarboxylase (90); glutamic dehydrogenase (29, 125); glutamic-pyruvate transaminase (89, 93, 126); glutamic-oxalacetate transaminase (89, 91).

particularly fruitful approach would appear to be possible: correlation of function (absorption) along the length of the intestine and in different physiological states with the altered enzyme levels which occur in these two situations. It is possible that this correlative approach would indicate the nature of the enzymic systems more intimately concerned with the active absorptive processes.

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Enzymatic histochemistry of gastrointestinal mucous membrane[1]

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Enzyme histochemistry deals with the characterization of enzymes in their natural locations within tissues and cells. It therefore aims to describe biochemical and morphological phenomena each in the terms of the other.

The application of morphological enzyme histochemistry to gastroenterology is scarcely more than a decade old and it is fair to say that recent progress in this direction has been conditioned primarily by the development of new methods. By way of illustration, when the late Professor George Gomori spoke on the histochemistry of gastrointestinal mucosa in 1952, at the Fifth Conference on Gastrointestinal Cancer, only five enzyme methods were included in his dissertation (1, 2). Today there are satisfactory histochemical methods for at least a score of enzymes, representing a

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fairly wide general coverage of enzyme systems, e.g., esterases, phosphatases, carbohydrases, phosphorylase, aminopeptidase, and a variety of oxidizing and reducing enzymes (3).

The enzyme, perhaps more than any other single biological constituent characterizes living matter and its functions. In the digestive tract, the chemical changes incident to digestion and absorption of the rather stable substrates available as foodstuffs are accomplished with the aid of specific enzymes which catalyze cleavage of large molecules, group transfer, oxidation-reduction and addition or removal of phosphate, water, carbon dioxide and ammonia. In more descriptive terms, the enzymes located within the gastrointestinal mucosa are concerned with secretion, absorption, active transport and the energy-producing mechanisms coupled to these processes. Their distribution can be approached in two ways. The first is by an "enzyme survey", which represents a harvest of descriptive data accruing from a systematic application of available methodology. The second is by a consideration of the integrated enzyme equipment of specialized cell types in different regions—with attempted physiologic interpretations. We prefer the "enzyme survey" at present, because histochemical information relating to a number of specific cell types populating the gastrointestinal mucosa is still fragmentary, and because the specificity and function of some of the enzymes we are able to demonstrate is not completely understood. As already indicated, the principal emphasis in enzyme histochemistry is on the enzymatic characterization of morphologic elements (endoenzymes). Therefore, once digestive enzymes (exoenzymes) have been discharged from their parent cells to act on substrates in the lumen of the gut, they are beyond the scope of the histochemist, and properly lie in the domain of the biochemist.

The present review describes a selected series of histochemical reactions which have been applied to mucous membrane of the gastrointestinal tract. I have not attempted to be all inclusive, but rather to illustrate for the gastroenterologist the possibilities offered by enzyme histochemistry for experiment and study in his discipline today.

ALKALINE PHOSPHATASE

More studies have been carried out on the histochemical localization of alkaline phosphatase in intestinal mucosa than on any other enzyme. This is not surprising in view of the long-standing interest in the physiologic significance of alkaline phosphatase at this site, and the fact that the methods for alkaline phosphatase developed by Gomori and Takamatsu (1939) and Menten, Junge and Green (1944) inaugurated the present era of enzyme histochemistry and were pre-eminent for a time. Allowing for differences in technique, localization artefacts and substrate specificities, the metal precipitation and azo dye methods yield parallel results under most circumstances (4).

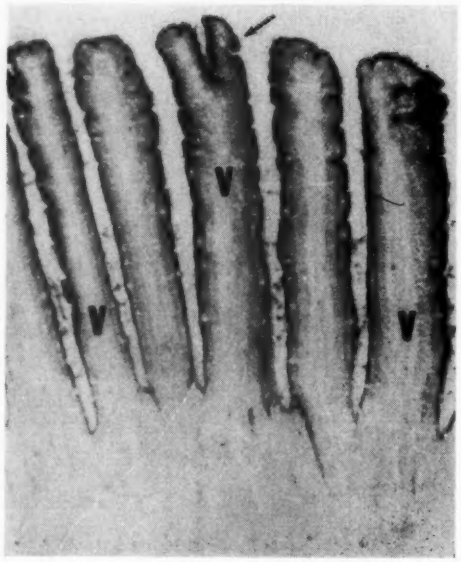

FIG. 1. Rat duodenum (cold acetone-fixed, paraffin embedded). Alkaline phosphatase localized to luminal border of epithelial cells lining villi (*v*). Gomori's calcium-cobalt method.

In the small intestine, alkaline phosphatase activity in columnar epithelial cells lining villi is very intense, and shows a bilaminar distribution in the striated border and in the underlying homogeneous portion of the apical cytoplasm. In an attempt to account for this staining pattern, Johnson and Kugler (5) have offered the ingenious explanation that 'phosphorylation of glucose occurs in the superficial part of the cell during absorption and then undergoes hydrolysis in a deeper part of the cell before passing into the blood stream. Dense aggregations of alkaline phosphatase also occur in the Golgi apparatus of intestinal absorptive cells, particularly in cells situated along the sides of villi. Inasmuch as the Golgi region has long been regarded as a center for cellular synthetic and secretory activities on cytological grounds, Deane and Dempsey (6) have suggested that the alkaline phosphatase in the Golgi zone may be destined to be secreted rather than playing a role in intracellular metabolism. Another possibility is that the alkaline phosphatase in the Golgi zone, as distinguished from that in the striated border of the cell, may be associated with the dephosphorylation of absorbed lipids (7).

In the rat, cat and dog, and probably also in other species, a gradient of decreasing alkaline phosphatase activity exists from the pylorus to the ileocolic valve (5, 8, 9). Histochemically, this correlates with an intense, bilaminar, cytoplasmic staining reaction, with no variations between cells at the tips and bases of villi in the proximal two-thirds of the small intestine, and only small, scattered areas of staining at the tips of villi in the distal third of the intestine (5). Species differences are also evident, in that intestinal epithelium of the rat gives a stronger reaction than either the dog or cat.

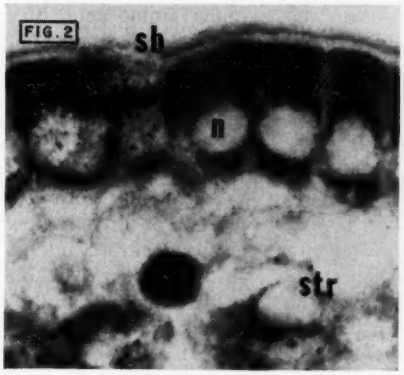

FIG. 2. Rat duodenum (cold calcium-formalin fixation, frozen section). Ali-esterase activity is distributed diffusely throughout the cytoplasm of enterocytes, and in droplets and granules of the Golgi apparatus (*arrows*). Striated border (*sb*), nuclei (*n*) and fibrous stroma of villus (*str*) are unstained. Naphthol-AS acetate—Fast blue salt BBN azo dye method.

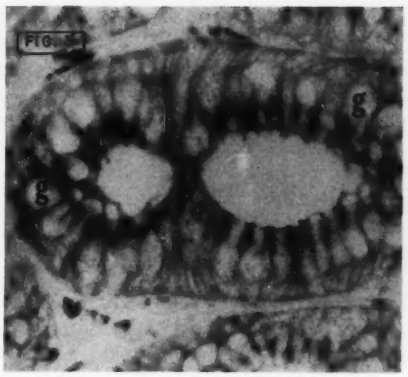

FIG. 3. Human colon (cold calcium-formalin fixation, frozen section). Intense ali-esterase activity in apical cytoplasm of goblet cells (g) lining a tubular gland. Naphthol-AS acetate—Fast blue salt BBN azo dye method.

Evidence from biochemical studies indicates that intestinal alkaline phosphatase is subject to quantitative variations related to diet and starvation. Thus, alkaline phosphatase activity of small intestine from rats fed a high-protein or high-fat diet is significantly higher than from animals fed a high-carbohydrate diet (10). Intestinal alkaline phosphatase is composed of a non-adaptive fraction which remains reasonably constant even after periods of prolonged fasting (up to 12 days), and a second adaptive portion which responds relatively quickly to the ingestion of fat and to fasting (11). Stenram (12) attempted to duplicate these findings using histochemical methods, but was unable to detect differences in intestinal alkaline phosphatase in groups of rats fed for 3 months on iso-caloric diets, high in carbohydrate, fat or protein. Recently, Lojda and Fábry (13) have succeeded in demonstrating a marked increase in alkaline phosphatase localized to the striated border and Golgi zone of jejunal enterocytes, in rats intermittently starved for 30 weeks. Nutritionally induced enzymatic adaptation, however, must be marked, before it can be recognized histochemically.

Histochemical procedures are well suited to the study of enzyme changes accompanying cellular differentiation during ontogenesis. In the pig embryo, alkaline phosphatase is highly active during the early stages of intestinal morphogenesis, then becomes inactive, but recovers activity just before functional differentiation of intestinal epithelial cells (14). In the chick, alkaline phosphatase accumulates slowly in the duodenum from 9 to 17 days of embryonic life, then rises rapidly to a peak in the striated border of duodenal enterocytes just after hatching (15).

The histochemical distribution of alkaline phosphatase in the colon is subject to marked species differences (16). The cat is foremost, giving a heavy staining reaction in all parts of the large intestine; the dog is a close second. In the rat, the striated border is found consistently negative over the whole length of the large intestine. Generally, alkaline phosphatase is not present in regions of the large intestine where a striated border is not obvious (16). In the stomach, only scattered glands show alkaline phosphatase activity (4). Positive cells are distributed along the epithelium of glandular crypts in the cat, whereas in man, they are concentrated in the surface epithelium. Blood vessels in the lamina propria stain intensely at all sites.

ACID PHOSPHATASE

Acid phosphatase has a widespread distribution in gastrointestinal mucosa; the following sites of activity are demonstrable by a post-incubation coupling technique using 6-benzoyl-2-naphthyl phosphate (17). In the rat stomach parietal cells stain intensely, while other cell types show negligible activity. In the duodenum, epithelial cells lining the glands of Lieberkühn, and to a lesser degree surface epithelial cells, possess strong acid phosphatase activity in apical portions of their cytoplasm. Goblet cells are negative, whereas epithelium of Brunner's glands reacts intensely. In the colon, surface epithelium and that lining the intestinal glands have moderate activity, but goblet cells fail to stain.

With Gomori's lead precipitation method, Deane and Dempsey (6) demonstrated acid phosphatase activity in the Golgi region and cuticular border of intestinal epithelial cells in the mouse, whereas corresponding cells in the guinea pig contained only small amounts of the enzyme. Burt, *et al.* (18), using the postincubation coupling azo dye method (17), have established that acid phosphatase localized to the cuticular border in the mouse duodenum is resistant to fluoride.

ALI-ESTERASE

It is difficult to give a simple description of the distribution of ali-esterase in gastrointestinal mucosa, inasmuch as the staining pattern obtained at most sites results from the combined activities of several closely related enzymes with overlapping substrate specificities and differing sensitivities to inhibitors (19). Differences in localization in homologous tissues are also encountered from one species to another.

In the stomach, the most intense esterase activity occurs in mucosa of the cardia and body. When beta

naphthyl acetate is employed as a substrate, most of the rabbit's gastric glands give a strongly positive reaction for esterase, whereas in the dog, staining occurs predominantly in surface epithelium and declines in intensity below the level of mucous neck cells. In guinea pig and man, the greatest esterase activity is found at the base of gastric glands. Parietal cells are more intensely stained than zymogenic cells in the rabbit and in man (20).

In the duodenum, epithelium covering villi stains prominently in rat, rabbit and guinea pig, when alpha and beta naphthyl acetate are used as substrates, the heaviest staining occurring in supranuclear cytoplasm and cuticular borders. Brunner's glands give a faint or irregular esterase reaction. Gomori (21) found that with Naphthol-AS acetate as substrate, there is a complete reversal of the foregoing picture in the rat, so that duodenal villi stain moderately, and Brunner's glands stain intensely. With the rabbit, on the other hand, the picture obtained with various substrates is identical. The cat duodenum stains poorly by all methods. The mouse falls between the rat and rabbit; with alpha naphthyl acetate, the villi stain markedly and the Brunner's glands moderately, while with acetylated Naphthol-AS, the villi stain moderately and Brunner's glands markedly (19). Recently, we have demonstrated with acetylated Naphthol-AS that in the rat, duodenal enterocytes contain an esterase in the Golgi region which is resistant to fluoride, whereas esterase localized to the apical cytoplasm and cuticular border is inhibited by fluoride.

Colonic mucosa of rat, guinea pig, rabbit, dog and man contains esterase localized in varying degrees to mucosal glands and surface epithelium (See figs. 2 and 3.)

The esterases of gastrointestinal mucosa appear at early stages of ontogenesis, e.g. as early as 45 mm CR-length, in man (22). In the chick, nonspecific esterase in the duodenum shows a slight rise between 9 and 13 days of embryonic life, increases gradually to 17 days, and markedly from that time to 3 days posthatching, when a plateau is reached (23). Recently, histochemical methods have been successfully combined with starch gel electrophoresis of tissue homogenates to produce zymograms, which demonstrate tissue protein fractions possessing similar enzymatic specificity (isozymes). Zymograms of a tissue prepared at successive stages in development generally reveal increasing numbers and amounts of esterase. However, the order of acquisition of new esterases during ontogenesis is not the same for all tissues, nor is there necessarily a steady increase in enzyme activity with advancing development (24). Important applications of this technique to problems in gastroenterology can be expected in the future.

LEUCINE AMINOPEPTIDASE (LAP)

Leucine aminopeptidase, which is present in the intestinal secretions of most animals, and intracellularly in a number of tissues, hydrolyses peptides having a free alpha-amino group on a terminal leucine or other alpha amino acid. Since the main specificity requirements of this enzyme are furnished by the leucine residue, the favorable chromogenic potentialities of synthetic substrates such as L-leucyl-β-naphthyl-amide (25) and L-leucyl-4-methoxy-2-naphthylamide (26) can be exploited in appropriate histochemical methods (27, 28, 29).

The stomach of rat and man is devoid of LAP activity, except for that present in small vessels and capillaries. In contrast, intense LAP activity localized to the striated border of columnar epithelial cells can be found in all segments of the small intestine (29). Brunner's glands are entirely negative. however. Colonic mucosa in man stains irregularly, with moderate to weak activity in patches of surface epithelium and some crypts. In the rat, colonic mucosa fails to develop a staining reaction, even after prolonged incubation in substrate medium.

SUCCINIC DEHYDROGENASE

Since the introduction of two new tetrazolium salts (Nitro-BT (30) and MTT (31)) as histochemical reagents in 1956, it has become possible to localize succinic dehydrogenase activity to intracellular organelles in suitably prepared tissue sections (3).

In the stomach (rat, mouse, guinea pig and man), parietal cells, which are located in the middle portions of gastric glands, stain intensely. Under the highest magnifications of the light microscope, deposits of formazan can be related to the numerous mitochondria which are present, and in lesser degree to intervening cytoplasm (32). Frequently, a system of unstained, branching intracellular canaliculi can be visualized in parietal cells, against the blue-staining background provided by enzymatically active cytoplasmic components. When the period of incubation is extended, chief and mucous cells stain lightly.

It is generally agreed that the parietal cell secretes hydrogen and chloride ions, and water, but does not participate in the synthesis of the protein constituents of the gastric juice. Anatomically, the formation of hydrochloric acid probably occurs in or on the boundary wall of the intracellular canaliculus. The various metabolic reactions of the parietal cell, including those concerned with production of energy for acid secretion, appear to be of the same character as are found in tissue cells generally, i.e. reactions of the tricarboxylic acid cycle, those involving the coupling of oxidative processes, and the production of high energy phosphate bonds (33). The actively secreting parietal cell has a high rate of oxygen consumption, which correlates with a rich complement of oxidative enzymes and a heavy population of mitochondria with exceptionally well-developed cristae and dense ground substance (34).

Some interesting histochemical studies have been carried out on succinic dehydrogenase activity in parietal cells following the administration of drugs which either depress or stimulate acid secretion. Telkkä and Kuuistos

(35) found that atropine and scopolamine did not modify the succinic dehydrogenase activity in the gastric mucosa of rat and mouse within 30 to 120 minutes of their administration. Pilocarpine and especially neostigmine caused an increase in the enzymatic activity of parietal cells of the rat and mouse; this increase could be seen within 10 minutes, and was also present in adrenalectomized rats. Villarreal and Burgos (36) observed in the rat that within one hour after the administration of acetyl-β-methylcholine (Mecholyl) the mucosa became thickened and the enzymatic activity of the parietal cells was much increased. Histamine produced a similar effect in the frog. How this rapid increase in succinic dehydrogenase activity, coincident with drug-induced acid secretion, comes about is not known; activation of latent enzyme would seem to be a likely possibility. (See fig. 4.)

In the rat and dog, epithelial cells lining the duodenum show an intense reaction for succinic dehydrogenase. The intracellular pattern of staining closely parallels the distribution of mitochondria. (See fig. 5.) In the jejunum and ileum, absorptive epithelium covering villi is more active than that lining crypts (32). Peyer's patches stain faintly.

Colonic epithelium of the rat gives a uniformly intense reaction, aside from mucous vacuoles which are negative. In the human colon, the nonmucoid cells at the base of the crypts give the most intense staining, particularly in periluminal cytoplasm. Surface epithelium stains less intensely and here the activity tends to be localized to the basal portion of the cell (37).

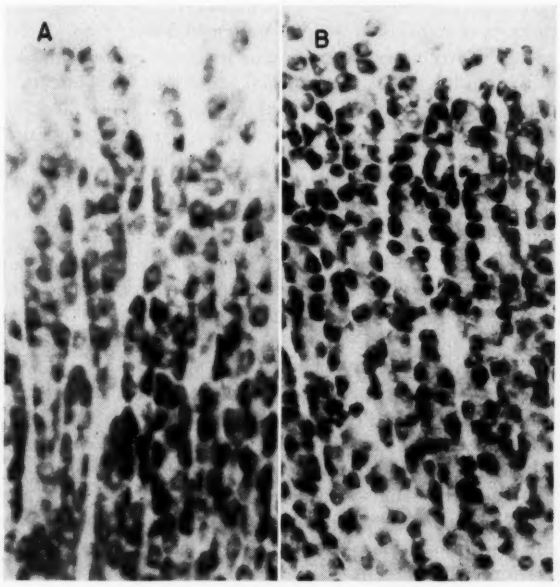

FIG. 4. Rat stomachs (cryostat sections of unfixed tissue) processed under identical conditions. Control animal (*A*) fasted for 24 hours. Experimental animal (*B*) received acetylcholine, 2 mg/100 gm body weight, subcutaneously, 15 min. before sacrifice. Note the increase in succinic dehydrogenase activity in parietal cells. Nitro-BT method.

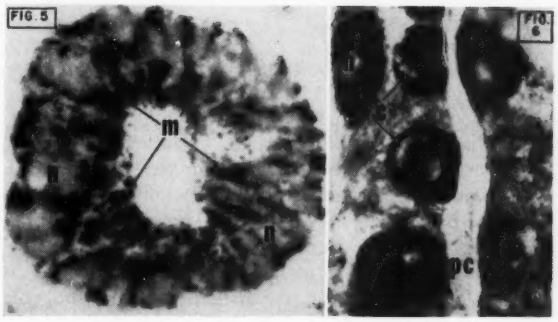

FIG. 5. Rat duodenum (cryostat section of unfixed tissue). Cross section through crypt of Lieberkühn showing succinic dehydrogenase activity associated with mitochondria (*m*). Cell nuclei (*n*) are unstained. Nitro-BT method.

FIG. 6. Human stomach (cold calcium-formalin fixation, frozen section). Parietal cells (*pc*) display intense lactic dehydrogenase activity. Intracellular canaliculi (*c*) and nuclei (*n*) are unstained. Nitro-BT method.

OTHER DEHYDROGENASES

The successful use of Nitro-BT for the cytochemical localization of succinic dehydrogenase subsequently led to the study of other dehydrogenase systems with this reagent. Appropriate modifications in the substrate mixture, involving the substitution of different substrates and the addition of cofactors, have made it possible to selectively demonstrate DPN and TPN diaphorases and certain DPN-linked and TPN-linked dehydrogenases in tissue sections (38, 39).

In the rat stomach, there is intense DPN diaphorase activity in surface epithelium and parietal cells throughout the entire extent of gastric glands. Only zymogenic cells at the base of glands are weakly stained (38). On the other hand, parietal and zymogenic cells give a feeble reaction for TPN diaphorase. At an intracellular level, DPN diaphorase activity is principally localized to mitochondria, whereas TPN diaphorase activity tends to be distributed throughout the cytoplasm (39). Concerning the distribution of various DPN-linked dehydrogenases in rat stomach, lactic and malic dehydrogenases are co-extensive with DPN diaphorase, while other specific dehydrogenases have a more restricted distribution. Thus, β-hydroxybutyric dehydrogenase activity is only present in the surface epithelium and the superficial half of glands, while ethanolic and glutamic dehydrogenases are limited exclusively to the surface epithelium (38). (See figs. 5 and 6.)

In colonic mucosa of man, the most intense DPN diaphroase activity occurs in surface epithelium and at the base of crypts. With TPN diaphorase, surface epithelium also stains relatively intensely, but cells at the base of crypts do not (40).

XUM

OTHER ENZYMES

The histochemical distribution of several other enzymes in gastrointestinal mucosa will be considered only briefly, because time limitations preclude a more detailed discussion at this juncture. Cytochrome oxidase, demonstrated with a new reagent for the G-Nadi reaction (4-amino-1-N,N-dimethylnaphthylamine), is abundant in parietal cells, absorptive cells of the small intestine, and colonic epithelial cells (37, 41). β-Glucuronidase also has a ubiquitous distribution, and is found in parietal cells and to a lesser degree in zymogenic cells of gastric glands, in columnar epithelium of small intestine, and in nonmucous cells of the large intestine (42). Monoamine oxidase is involved in the metabolism of some of the "biogenic amines" but may also serve a "detoxicating function" in the gut. Epithelial cells lining the small intestine contain the largest amounts of this enzyme with distinctly lesser quantities in chief cells of the gastric fundus and in colonic mucosa (43). Carbonic anhydrase, demonstrated by Häusler's modification of Kurata's method, has been reported as occurring in gastric parietal cells at different stages of development in several species (44). These results, however, must be accepted with caution until more information is available on the validity of the staining procedure employed (3, 45).

APPLICATIONS TO DISEASE IN MAN

The development of reliable methodology and the establishment of norms have enabled gastroenterologists and pathologists to extend the application of enzyme histochemistry to gastrointestinal diseases in man. This work will be summarized briefly, in relation to lesions of the stomach, small intestine and colon.

Stomach. Subacute and chronic gastric ulcers show enzyme changes comparable to those encountered in a healing wound, with high concentrations of alkaline phosphatase and leucine aminopeptidase in proliferating fibrous tissue forming the ulcer floor (46–48). In chronic gastritis with intestinal metaplasia, a positive correlation has been found between the severity of the process and alkaline phosphatase activity, so that in extreme cases there may be a strong resemblance to small intestinal epithelium, both morphologically and enzymatically (46, 49). Similarly, Wattenberg (50) found high leucine aminopeptidase activity in intestinal metaplasia of the stomach, and in approximately one-third of gastric carcinomas, whereas if present at all, extremely low activities of this enzyme exist in normal gastric mucosa. In the normal small intestine intense aminopeptidase activity occurs at the periluminal border of mucosal epithelial cells. These findings suggest a metabolic relationship between gastric intestinal metaplasia and small intestinal epithelium, and also support the concept that intestinal metaplasia in the stomach is a precursor to malignancy (50). On the other hand, in "atrophic gastritis," "lipase" activity is greatly diminished or absent, due to the loss of normal body glands and their replacement by metaplastic epithelium (51).

Carcinomas of the stomach generally contain lower levels of hydrolases than their normal tissues of origin, although of course exceptions can be found to this statement. In one series (52), 6 of 10 carcinomas of the stomach fixed in cold acetone were devoid of esterase activity, while four showed slight activity. In another series reported by Manheimer and Seligman (4), alkaline phosphatase was present in scattered collections of tumor cells in only 4 of 15 gastric carcinomas fixed in alcohol or cold acetone. Stromal blood vessels in the tumor bed were also relatively deficient in this enzyme, in comparison to blood vessels supplying normal mucosa. Acid phosphatase appears to be more plentiful, inasmuch as Reiner *et al.* (53) found that 2 of 4 adenocarcinomas of the stomach stained rather intensely for this enzyme. Leucine aminopeptidase activity was noted by Wattenberg (50) in 7 of 20 carcinomas of the stomach. In well differentiated areas, where tumor cells reproduced glandular structures, staining tended to occur at the luminal border, whereas poorly differentiated carcinoma cells stained diffusely. There was no correlation between the degree of cellular differentiation and the presence or absence of aminopeptidase activity. Leucine aminopeptidase occurs with considerable frequency in the stroma of carcinomas, but merely reflects the presence of proliferating fibroblasts therein, and is unrelated to invasive properties of the neoplasm (48).

Oxidases and dehydrogenases have not been studied extensively as yet in gastric tumors. DPN diaphorase activity is as high as in normal gastric mucosa, shows little variation from case to case, and tends to be localized to mitochondria and small cell membranes. Succinic dehydrogenase and TPN diaphorase activities are far below those encountered in normal gastric epithelium (54).

Small Intestine. Bolt *et al.* (55) have performed peroral biopsies of small intestines in patients with malabsorption syndromes and observed that alkaline phosphatase is deficient in the "brush border" region in primary sprue. This loss of alkaline phosphatase may correlate with the absence of microvilli from intestinal absorbing cells recently demonstrated by electron microscopy in cases of non-tropical sprue in relapse (56, 57).

Colon. The pattern of hydrolases occurring in carcinomas of the colon differs only in a few respects from that encountered in corresponding lesions of the stomach. The majority of adenocarcinomas of the colon lack appreciable esterase or alkaline phosphatase activity (4, 47, 52). Reiner *et al.* (53) studied 10 carcinomas of the colon for acid-phosphatase activity, and observed lower concentrations than in epithelium of normal mucosa in 4 instances, amounts equal to the latter in 4 others, and higher levels in neoplastic than in nonneoplastic epithelium in only two. Differences in intensity of the staining reaction between various specimens or within the same specimen could not be correlated with their histologic grades of differentiation. Leucine aminopeptidase is not a fea-

ture of the epithelial component of colonic carcinomas, although it may be irregularly distributed in the stroma of these tumors (48).

Several histochemical studies of oxidative enzymes in benign and malignant proliferative lesions of the large intestine have shed light on the metabolic changes involved in the sequence from focal mucosal hyperplasia to benign adenomatous polyp, adenoma malignum and finally to invasive carcinoma (37, 40, 54, 58). In benign focal hyperplasia there is low succinic dehydrogenase, α-glycerophosphate dehydrogenase and monoamine oxidase activity. DPN diaphorase activity is not increased, but elevation of TPN diaphorase activity may occur. Several patterns of reaction are encountered in benign adenomatous polyps, but none of these duplicate the rather characteristic findings in carcinoma. Polyps feature the presence of high succinic dehydrogenase, α-glycerophosphate dehydrogenase and monoamine oxidase activity, the most intense staining occurring in the deeper portions of the atypical glands (37, 40). In contrast, invasive carcinomas and malignant polyps display high DPN and TPN diaphorase activity and low cytochrome oxidase, succinic dehydrogenase, and monoamine oxidase activity. Of the various DPN-linked dehydrogenases, lactic, malic and β-hydroxybutyric dehydrogenases show relatively high activity, while glutamic and ethanolic dehydrogenase activities are weak or absent (54). These features are best observed in cells at the invading margin of the tumor.

Important disorders of the gastrointestinal tract, such as ulcerative colitis, regional enteritis, and secondary malabsorption states have, of necessity, been omitted from this presentation for want of information. It is hoped that a continuation and extension of histochemical studies by a growing body of interested workers will soon fill this gap.

CONCLUSION

The studies cited indicate that histochemical techniques when applied to gastroenterology, have the advantage of revealing the distribution of enzymes among various specific types of cells in a mixed population, and in some instances of localizing enzyme activity to intracellular organelles. Accordingly, certain problems in embryology, comparative biochemistry and experimental physiology requiring the characterization of enzymes in minute structures, are ripe for such an approach. In other areas of gastroenterology, histochemical methods enhance the significance of purely quantitative biochemical data, by permitting a closer correlation of chemical results with functional and structural tissue units. For example, mucosal epithelial cells which are morphologically similar, quite commonly show otherwise unsuspected gradients of enzyme activity, presumably related to differences in their functional state. It is anticipated that future advances in histochemistry will contribute greatly to our understanding of the location of enzymes and the flow of substrates in epithelial cells of the gut, under normal and pathologic conditions.

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Fucose and sialic acid in glycoproteins of the mucus of the digestive tract

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The mucus of the digestive tract seems to have essentially two main physiological functions, namely, to influence the mobility of the contents of the digestive tubes and to exert a protective action on the mucosa of the tract. The protective effects are partly due to mechanical properties of the mucus and partly to specific chemical antibacterial and antiviral actions. It is clear that this physiological function of the mucus is dependent on characteristic physico-chemical and ultimately on chemical properties of certain mucus constituents. Mechanical properties of the mucus, which appear significant from the functional point of view, namely high viscosity and stringiness, usually are associated with highly polymerized substances. It has been found as far back as 1946 (1) that the highly viscous mid-cycle cervical mucus contains a polysaccharide protein complex which accounts for almost all of its nondialysable carbohydrate. This substance, or group of substances, appear to be the constituent of this type of mucus which could be responsible for its very high viscosity. The carbohydrate part of this polysaccharide protein complex has been shown to be a heteropolysaccharide consisting of galactose, hexosamine and fucose in proportions which previously were found by other investigators in substances with blood group activity. These findings suggest that polysaccharide protein complexes of the same type are essential constituents of the mucus of the digestive tract as well as of that of other organs.

An investigation of the chemistry of the mucus of the digestive tract encounters several difficulties apart from those inherent in the structural complexity of compounds representing complexes of highly polymerized heteropolysaccharides with several sugar constituents and protein residues. One of the difficulties to cope with, particularly in clinical work on mucus, comes from the fact that mucus samples which can be directly obtained from the organism almost always represent a mixture of secretions of different glands. Saliva, for instance, contains mucus from 3 different glands and, as Tsuiki *et al.* recently reported (2), glycoproteins from the submaxillary and sublingual glands of beef contain glycoproteins of completely different composition. The same situation prevails in the case of the gastric and duodenal mucus. The first contains at least two different secretions, namely that of the surface epithelium and that of the chief neck cells; the latter is a mixture of three completely different secretions: the pancreatic juice, the bile and the mucus of the Brunner gland. It is clear that an investigation of the chemistry of mucus in these cases ultimately will require surgical isolation of the individual secretory organs. Another difficulty which comes up in these cases is due to effects of enzymes secreted simultaneously with the mucus. It has been shown, for instance, that a jelly-like mucus originating in the gastric surface epithelium (3, 4) is readily depolymerized during the incubation of the gastric juice at 37° even at slightly alkaline pH. Indirect evidence was obtained that in the duodenal fluid carbohydrate protein complexes can lose part of their protein moiety by enzymic activities of the fluid. This situation may make it impossible without further experi-

mental devices to investigate the chemistry of the mucus as originally secreted by the glands. In the lower sections of the intestine the situation appears less complicated as far as multiplicity of the origin of the mucus is concerned. The main difficulty here is the paucity of the material under physiological conditions. This is the reason why most of the investigations in this region were concerned not with the chemistry of the mucus actually secreted by the gland, but with the chemistry of carbohydrate protein complexes obtained from the whole mucosa or its epithelium. It is obvious that this procedure introduces a major source of error and confusion as the cellular and intercellular elements of mucus membranes may contain considerable amounts of polysaccharides very similar in composition to those present in mucus. A great number of data on the composition of mucus of the intestinal tract found in the literature, therefore, are derived from mucus material which was obtained after stimulation of the secretory process by either parasympatico-mimetic drugs or strong irritants of mucus membranes. It is often difficult to decide how far mucus obtained under these conditions really corresponds to that secreted under physiological conditions (5).

This brief report deals with certain aspects of the structure of the carbohydrate of glycoproteins found in mucus secreted by certain parts of the digestive tract. Particular emphasis will be laid on the relation of two characteristic constituents of the carbohydrate, namely fucose and sialic acid to each other as well as to certain functional and pathological changes of the mucus secreting glands.

GLYCOPROTEINS OF THE MUCUS OF THE DIGESTIVE TRACT

The most extensive investigations of glycoproteins of the digestive tract were so far concerned with those of the gastric mucus and mucus of the duodenal fluid. These forms of mucus are readily available from clinical material and in the case of the stomach can also be obtained with relative ease from Heidenhein pouches in dogs. As far as the small and large intestines are concerned, only very few scattered data are available in the literature. Some of these data, however, when considered in the proper context are of great interest and highly suggestive. The investigation of the glycoproteins of the mucus secreted by the stomach mucosa were mainly concerned with the isolation by chromatographic and electrophoretic methods of more or less well-defined glycoprotein fractions from gastric juice and from mucus secreted in Heidenhein pouches of dogs after stimulation (6–8). It has been found that the glycoprotein represent throughout a mixture of numerous fractions. A more detailed chemical analysis, however, of the carbohydrate of these fractions so far has not been reported. Werner (9) and Glass *et al.* (10) reported analytical data on glycoprotein fractions obtained from gastric juice by fractionation with TCA acetone. The data, however, do not appear completely adequate as the glycoprotein fractions apparently represent mixtures of a hexuronide containing hexosamine and a neutral polysaccharide fraction containing hexose, fucose, hexosamine and sialic acid. In the experiments of Glass and his associates the hexuronic acids had not been determined with sufficient accuracy and therefore it does not appear possible to evaluate the amount of hexosamine combined with the neutral polysaccharide. Werner's data are difficult to interpret from our point of view because fucose has not been determined in his glycoprotein fractions from gastric juice.

The mucus of the duodenal fluid appears in many ways to represent a simpler system from the chemical point of view than the gastric mucus in spite of the fact that it is derived from three different organs. The chemistry, therefore, of the duodenal mucus will be first discussed, particularly as analytical studies in this case appear to be in a more advanced stage than in the case of the gastric mucus.

Glycoproteins of the duodenal fluid and in the mucus of the small intestine. For the investigation of the glycoproteins of the duodenal fluid it proved of particular advantage to precipitate quantitatively the substances with 9 parts of a mixture of equal parts ethanol and benzene which removes the bile pigments from the precipitated glycoproteins and makes it possible to analyze them without further preparation. The glycoproteins from the duodenal fluid of children under these circumstances are completely soluble in water. In adults, in some individuals an insoluble residue remains, containing carbohydrate and protein. The first can be quantitatively brought in solution by heating at 90°C with 5% TCA for 6 hours. In children affected with cystic fibrosis of the pancreas, a systemic disease of mucus secretions, up to one-half of the carbohydrate material precipitated by ethanol benzene also remains insoluble in water and must be extracted for analytical purposes with TCA. The material which dissolves in water is to about 80%–90% nondialyzable.

After addition of TCA to the water solution a small part, 10%–15%, of the carbohydrate is precipitated together with protein material. The ratio carbohydrate to protein in those precipitates in normal children and adults is about .1 to .15. This material will be referred to as TCA insoluble glycoprotein, whereas the material remaining in solution as TCA soluble glycoprotein. The TCA soluble material of the duodenal fluid from children as well as adults was shown to contain two different glycoproteins which differ in the composition of the carbohydrates and could be separated by precipitation with two parts of ethanol. The carbohydrate of the precipitated glycoprotein contained as constituents, identified by paper partition chromotography with several solvents, galactose, glucosamine, galactosamine, fucose and a substance giving the color reactions of sialic acid, (Fraction I.) Carbohydrates in the supernatant of the ethanol precipitation obtained after removal of the ethanol from the solution and dialysis of the neutralized solution contained galactose and mannose in about equivalent amounts, glucosamine, very little, if any, galactosamine, fucose and sialic acid (Fraction II.)

The characteristic structural feature in both types of

TABLE 1. *Composition of the TCA-Soluble Glycoprotein of Pancreatic Juice and Bile of Adults*

Exp. No.	TCA Soluble Glycoprotein fraction	Total Volume of Juice *cc*	Galactose	Mannose	Glucose	Total Hexose	Fucose	Hexosamine	Sialic Acid	Total Carbohydrate	Total Protein *mg%*	Protein % of *Fraction*
1	Glycoprotein from pancreatic juice of the patient with common duct obstruction due to stone	660	2.25	0.96	0	3.2	1.6	3.56	1.1	9.4	20.8	69.0
2	From pancreatic juice of the patient with acute pancreatitis	330	2.1	0.79	0.32	3.2	1.44	2.79	0.98	8.2	20.4	71.0
3	From pancreatic juice of the patient with Ca of pancreas	475	0.31	0.24	0.05	0.6	0.25	0.55				
4	Bile from a patient with duct obstruction due to stone	55	0.85	0	0		0.4	0.89			4.6	

All values in mg/100 cc of fluid

polysaccharides appeared to be the stoichiometric molar ratio between fucose and total hexosamine of about .5. On the other hand, the ratio sialic acid to hexosamine was very much higher in the more soluble glycoprotein than in the less soluble. If hexose instead of hexosamine was taken as reference point, then the ratios of fucose to hexose and of sialic acid to hexose varied from one glycoprotein to another in the opposite sense. The carbohydrate of the TCA insoluble glycoprotein had the composition of a mixture of carbohydrates of Fractions I and II.

The presence of two different glycoproteins in the duodenal mucus raised the question whether both are present in all of the constituents of the duodenal fluid. It has been known before that in some form, of mucus, for instance in mid-cycle cervical mucus, no mannose containing glycoprotein is present whereas in mucus from other sources varying amounts of this hexose had been found by Werner (9). For this reason, the TCA soluble glycoprotein of three samples of pancreatic juice[1] and the TCA soluble glycoprotein of one sample of human bile obtained during surgery were analyzed.

Pancreatic juice was obtained from patients with pancreatic fistulas. Two patients had been operated on for gallstones and a third for cancer of the pancreas. The juice was collected during a period of 12 hours and then kept frozen for several days until analyzed. As the juice was found not to contain any substances interfering with analytical procedures, the glycoproteins were fractionated immediately (without previous precipitation with benzene ethanol) by 4% TCA which produced an abundant precipitate. The concentration of glycoprotein in the supernatant of the TCA precipitation was so low that it was necessary to concentrate it for analysis. To avoid decomposition by the high concentration of TCA, the supernatant after separation was neutralized with solid $NaHCO_3$, dialyzed for 24 hours against frequently changed large volumes of distilled water at 4°C, and then concentrated *in vacuo* to about one-tenth of its original volume.

With bile the same procedure was applied as with pancreatic juice. The supernatant of the TCA precipitation, when concentrated after dialysis to one-tenth of its original volume, was found still to contain too much pigment material. To remove the latter, the concentrate was precipitated with nine parts of benzene-ethanol mixture and the precipitate treated like that from duodenal fluids.

TCA insoluble material from both sources was unsuitable for complete analysis because in the pancreatic juice the carbohydrate represented only 2% of the material and in the bile the TCA precipitate contained most of the bile pigment which interfered with our analytical procedures reaction. The results of the analysis of the TCA soluble material are listed in Table 1. As can be seen, the TCA soluble glycoproteins from pancreatic juice contain mannose and galactose fucose and hexosamine. The presence of galactose, mannose and fucose was also demonstrated by paper chromatography of hydrolysates of the TCA-soluble glycoproteins in three different solvents. The ratio mannose to galactose varied in different samples of juice between 0.4 and 0.8. The fucose to hexosamine ratio was about 0.5. On this basis it can be assumed that the pancreatic juice contains both glycoproteins I and II, with a higher proportion of II than prevails in the whole duodenal fluid. The TCA soluble glycoprotein of the bile, on the other hand, proved in this one case at least to be free from mannose. It is remarkable furthermore that the ratio total carbohydrates to nitrogen in the TCA soluble glycoproteins of the pancreatic juice does not significantly differ from this ratio as found in the TCA-soluble glycoproteins of the duodenal fluid. No data at present are available on the composition of pure secretion of Brunner glands in humans. Kent *et al.* (12) collected mucus from Brunner glands of rabbits obtained from surgical fistulas. They found in the mucus .4% of a carbohydrate which con-

[1] The author is greatly indebted to Dr. H. Dubilet of the New York University Medical School for the three samples of pancreatic juice which were analyzed.

tained hexosamine as one of the constituents. In addition, ester sulfates were found in a concentration of .8%. This suggests the presence of a sulfated polysaccharide. No determinations of hexuronic acid unfortunately were carried out in this investigation so that it is not possible to decide whether the sulfated polysaccharide is a polyuronide identical or related to the sulfated polyuronide found in gastric mucus. The test for hexauronic acid with carbazole H_2SO_4 was negative in duodenal fluids from children and adults. This, however, might be due to the dilution of the Brunner glands mucus by the mucus from the pancreatic ducts and bile.

Further subfractionation of the TCA soluble glycoproteins from duodenal fluid from children and adults (11) brought out certain structural peculiarities of these glycoproteins which on the basis of all the evidence now available might be of significance for the determination of the physical properties of these glycoproteins and also be related to their specific function in the body. When the two glycoproteins I and II were subfractionated with ethanol, two subfractions of I were obtained, which precipitated at 50% and 67% ethanol respectively (Subfractions A and B). These two subfractions differed in their ratio of fucose and sialic acid to hexose in such a way that the first ratio was lower and the second one higher in the more soluble fraction. The glycoprotein II was split into two subfractions by precipitation with 90% ethanol. The precipitate showed a significantly higher ratio fucose to sialic acid than the material remaining in solution which was analyzed after removal of ethanol in vacuo from the neutralized supernatant and dialysis with subsequent concentration to a suitable volume. When this last subfraction was further subfractionated with acetone, the precipitates obtained with 5 and 9 parts of acetone respectively again differed in their fucose to hexose ratio which in the less soluble subfraction was again much higher than in the more soluble subfraction. These data therefore indicate an inverse correlation of the fucose to hexose ratio and a direct correlation of the sialic acid to hexose ratio with solubility of glycoproteins of the duodenal fluid in ethanol and acetone water mixtures. These opposite effects of fucose and sialic acid on solubility of glycoproteins can be easily understood if we consider that both these constituents are present as end groups in the carbohydrate of the glycoproteins and therefore the hydrophobic methyl group of fucose and the hydrophilic free carboxyl group of sialic acid may have opposite effects on the attraction of water molecules by the surface of the glycoprotein molecules.

The chemical analysis (13) of glycoproteins of the duodenal fluid of children with cystic fibrosis of the pancreas (CFP) led to results which suggest that fucose and sialic acid play also an antagonistic role in the determination of the stability of glycoprotein molecules of the duodenal fluid. As was mentioned above, the precipitation of the duodenal fluid from children with CFP with ethanol benzene yields a precipitate which is partly water insoluble and from which all carbohydrate can be extracted by 5% TCA at 90°C (14). The analysis of this H_3O insoluble residue and of fractions of TCA soluble glycoproteins of the duodenal fluid of children with CFP revealed that the carbohydrate of the water insoluble fraction has essentially the same composition as the subfraction A precipitated with 50% ethanol. Compared with the corresponding subfraction A of the TCA soluble glycoprotein from the duodenal fluid of children without CFP, this water insoluble material and fraction A from children with CFP showed a higher fucose and lower sialic acid content. As the loss of water solubility takes place only after precipitation with organic solvents, the appearance of this water insoluble fraction is an expression of a higher liability to denaturation of the glycoprotein of the duodenal fluid in patients with CFP, and the latter therefore may be related to a simultaneous increase in fucose and decrease in sialic acid.

Glycoproteins of the mucus of the small intestine. A few data on the composition of glycoprotein in the mucus of the small intestine reported by Werner (9) suggested that the content in fucose and sialic acid of glycoproteins apart from its influence on the above-mentioned physicochemical properties of solubility and stability may also be related to functional changes of the secreting mucus gland. Werner studied the mucus which accumulated on the surface of the mucosa of the small intestine of a patient with an intestinal fistula. Because of small amounts of the material secreted under normal conditions, only incomplete analysis could be carried out. Much larger amounts of mucus, however, could be collected by local application of acetylcholine or prostigmine. The analysis of the glycoprotein of this "stimulation" mucus showed significant changes in the ratio of fucose and sialic acid to hexosamine in the sense that the ratio of fucose to hexosamine increased, after stimulation. Analytical data from this experiment are listed in table 2.

Glycoprotein of the mucus of the submaxillary gland of dog. It was clear that to obtain unequivocal results about a possible relation of the two end group constituents of mucus glycoproteins with various functional states, it would be of advantage to study changes in composition of mucus secreted from isolated organs under as much as possible standardized and reproducible conditions of stimulation and secretion and avoidance of secondary enzymic breakdown of glycoproteins. An investigation, therefore, was started in collaboration with C. Pallavicini and in conjunction with Dr. L. J. Cizek and S. Chien, of the Department of Physiology, of the College of Physicians and Surgeons, on the composition of saliva from the submaxillary gland of dogs under influence of different stimuli of varying intensity and duration. The results of one part of this investigation which is continuing seem

TABLE 2. *Composition of the Carbohydrate of the Glycoprotein of the Mucus of the Upper Ileum Before and After Stimulation by Acetylcholine*

	Hexosamine	Fucose	Sialic Acid
Before stimulation	9.3	2.1	
After stimulation	10.8	3.7	4.2

All values in % dry weight. Compiled from Werner (9).

clearly to demonstrate certain correlations between the content in glycoprotein of the submaxillary mucus and its output per minute, both dependent on dosage of pilocarpine, and variations in the content in fucose and sialic acid of the mucus carbohydrate. The variations in the content of these two constituents here again take place in opposite directions and here again, the two constituents appear to influence in the opposite sense the solubility of the glycoprotein of saliva in ethanol.

The experiments were carried out on dogs narcotized by injections of 33 mg/kg Nembutal. The ducts of submaxillary glands were cannulated with tephlon tubes and the saliva secreted after stimulation of the gland was collected for certain periods in test tubes with the rate of the outflow measured during the collection by counting drops per minute. After the collection of the sample was finished, the sample was immediately frozen by immersing in an acetone and dry ice mixture and kept frozen until it could be further processed.

The particulate matter of the mucus was removed by thirty minutes centrifugation at 8000 RPM in a Servall centrifuge and glycoproteins were quantitatively precipitated at 0° with 9 parts of ethanol. The precipitate was twice washed with ethanol and dissolved in water. Complete analysis of the sugar constituents of the glycoprotein were carried out by the colorimetric procedures used for this purpose in our laboratory (15). The sugars were identified by paper chromatography and in addition hexosamines by chromatography according to Gardell (16). The glycoprotein of the submaxillary mucus was found to consist of galactose, fucose and sialic acid, glucosamine and galoectosamine. The last two in a ratio of about 3 to 2. $\frac{\text{Total carbohydrate (TCH)}}{\text{Total glycoprotein}}$ [2] = about 0.7.

In the experimental series here reported, the saliva secretion was stimulated by intraveneous injection of a 0.1 % solution of pilocarpine. Three types of experiments were carried out. In the first series, the stimulation was started with a low dose and after the outflowing saliva had been collected during a certain time interval, a next higher dose was injected and again the collection continued until the output of saliva went down to a very low level, whereupon again, a still higher dose was injected. When the injected dose exceeded 2 mg pilocarpine, the outflow of saliva decreased sharply and at the same time, its viscosity increased very considerably. In a second series of experiments, the stimulation started with a medium dose, after which two samples were collected. The first immediately after the injection when the outflow of saliva was high, and the second during the time interval of lower output of saliva. When the latter dropped to a very low rate, it was increased by the injection of a dose of pilocarpine, lower than the initial one, and after a certain time interval, the medium dose was again injected and followed later by a still higher one. In a third type of experiment finally the stimulation was started with an optimum 4 mg dose of pilocarpine. The mucus was collected in two successive time intervals whereupon another injection of 4 mg followed to bring the outflow of saliva to a higher level. In table 3, the results of one of each type of such experiments are listed. It can be seen, first of all, that the very definite average stoichiometric relation of one appears between the sum of molecules of fucose and sialic acid (S + F) and the number of molecules of hexosamine in the carbohydrate of the glycoprotein.[3] On the other hand, the ratio between sialic acid and fucose changes continuously with the change in the intensity of the stimulus while the molar ratio of about one between S + F and hexosamine is maintained throughout the whole experimental series. It is clear, therefore, that fucose and sialic acid can replace each other as end groups, which probably are linked to the hexosamine of the glycoproteins. Although the ratio sialic acid to fucose varies with the doses of pilocarpine, there does not appear a definite correlation between this ratio and the size of the dose. Instead, there appears an inverse correlation between the ratio of S to F and two factors which could be related to the functional efficiency of the secretory process itself. The first is the concentration of the glycoprotein in the mucus, and the second, the output per minute. In *experiment 2*, the ratio S to F declines rapidly from 2.5 during the first time interval, to .5 in the third. This decline parallels the increase in the output per minute of glycoprotein as measured by its total carbohydrate and in sample 3, accompanies also an increase in the concentration of the glycoprotein. In *experiment 1*, the ratio S to F during the first three time intervals with low glycoprotein concentra-

TABLE 3. *Molar Ratios of Sugar Constituents of Glycoproteins of Saliva and Their Relation to Output and Concentration of Glycoproteins*

Exp. No.	Time Interval of Collection of Sample *min.*	Pilocarpine Injected at the Beginning Time of the Interval *mg*	TCH* Secreted *mg/min.*	TCH *mg/100 ml saliva*	F/Gal.	F/Hex.	S/F	(S+F)/Hex.
I	1.) 17	1.0	0.55	45.6	0.47	0.45	1.47	1.12
	2.) 48	0	0.07	28.8	0.41	0.30	1.80	0.86
	3.) 36	0.4	0.29	13.7	0.39	0.34	1.67	0.92
	4.) 14	1.0	1.54	55.2	0.80	0.61	0.51	0.95
	5.) 22	2.0	1.34	128.0	0.93	0.81	0.44	1.17
	6.) 31	4.0	5.08	188.0	0.91	0.74	0.47	1.11
II	1.) 33	0.3	0.12	39.6	0.31	0.28	2.5	0.98
	2.) 13	0.6	0.51	38.0	0.56	0.49	1.1	1.02
	3.) 3	1.0	1.14	79.5	0.86	0.56	0.52	0.95
III	1.) 8	4.0	2.78	148.0	0.53	0.46	1.0	0.94
	2.) 22	0	1.08	193.6	0.77	0.36	0.57	1.03
	3.) 7	4.0	4.05	168.8	1.11	0.30	0.37	0.91
	4.) 26	0	2.05	292.8	0.84	0.27	0.39	0.97

* TCH = Total carbohydrate = sum of anhydro residues of all sugar constituents plus one acetyl. F = Fucose; Hex = Hexosamine; Gal = Galactose.

[2] TCH calculated as the sum of anhydro residues of all sugar constituents plus one acetyl.

[3] Although the values for (S + F)/hexosamine vary from one sample to another between 0.84 and 1.17 the averages of samples in every one of the three experiments vary between 0.97 and 1.0.

tion and low output per minute is three to four times as high than in the last three samples in which both the output and the concentration increased to a very considerable extent. In *experiment 3* which was started with a dose of pilocarpine above optimal changes in the concentration and in the output did not run parallel. Here S/F markedly and continuously decreases with progressing time. It will be noted, however, that in every instance, the decrease can be either related to an increase in concentration or in output. The final result may depend which of the two factors, when moving in opposite directions prevails in its influence. These results indicate that during the secretion of saliva, the composition of the carbohydrate of glycoprotein is continuously changed by a mutual exchange of the two end group constituents fucose and sialic acid. This exchange may play a role in the regulation of physical properties of the excreted mucus, as well as the rate of output of saliva. It seems reasonable to assume that sialic acid as a charged molecule will tend to increase the binding of water by the glycoprotein and thus in turn influence the viscosity of mucus. The increase in number of charges accompanying the increase in the concentration of the glycoprotein may therefore represent a mechanism for the control of viscosity of the mucus. The relative decrease of sialic acid, on the other hand, and its substitution by fucose with increased output of saliva, can be also interpreted as a self-regulatory factor insofar as the decrease in the hydrophily of the glycoprotein may tend to decrease the rate of the output of fluid and thus prevent the exhaustion of the secretory ability of the gland.

INFLUENCE OF THE S/F RATIO ON CHANGES IN SOLUBILITY OF THE GLYCOPROTEIN OF SALIVARY MUCUS

The possibility to achieve a continuous and gradual change in the S to F ratio of the carbohydrate of the salivary glycoprotein suggested to test whether shifts in this ratio affect the solubility of the glycoprotein in ethanol in the same sense as it was found to be the case for the glycoprotein of the duodenal fluid. To this end, the four samples from *experiment* III and the sample 2 from *experiment* II of table 3 were subjected to fractionation with ethanol. In *experiment* III, three protein containing fractions were obtained with all four samples 1–4, which precipitated, at 50%, at 60% and at 67%, respectively. In the case of sample 1, with the highest S to F ratio, the first two fractions did not contain any significant amounts of carbohydrate, but 92% of the carbohydrate of the mucus were recovered in the third fraction. In sample 4 of *experiment* III, with the lowest ratio S to F, no significant carbohydrate was found in fractions one and three, whereas the fraction two precipitated at 60% contained 90% of the carbohydrate. In samples 2 and 3 of III, the carbohydrate was divided among fractions two and three. In sample 2 with the higher S to F, more appeared in fraction three. In sample 3, more appeared in fraction two. In this case, therefore, there appears a complete parallelism between the ratio S to F and solubility in the same sense as it was found also in the case of the mucus from the duodenal fluid. The S to F ratio in the individual etanol fractions was always identical with that for the whole glycoprotein material. This shows clearly that sialic acid and fucose form part of the same molecule and are not constituents of two different glycoproteins present in mixture. It appears highly improbable that the solubility of two different glycoproteins, one of which contains predominantly fucose and the other, sialic acid, should be identical.

The phenomena of the mutual substitution of sialic acid and fucose is by no means a characteristic of glycoprotein fractions in the mucus of the digestive tract.

The fractionation of nondialysable carbohydrate of male urine in our laboratory (17) by isopropanol precipitation, yielded three main fractions which differed by their solubility in isopropanol. The subfractionation of these three main fractions by continuous flow electrophoresis yielded eight subfractions which all differed significantly by their S to F ratio. Within every one of the three main fractions, the subfractions, showed an inverse correlation between the S to F ratio and the S to Hexosamine ratio.

Finally, determinations in our laboratory of the two ratios $\frac{\text{Fucose}}{\text{Hexosamine}}$ and $\frac{\text{Sialic acid}}{\text{Hexosamine}}$ in glycoproteins combined with various globulin fractions prepared from human plasma according to E. Cohn ($II_{1,2}$, II_3, III, III_0, IV_1, IV_4) also showed an inverse correlation between the two ratios in different fractions. This correlation was found in determinations on the total carbohydrate of the globulin fractions as well as in purified glycoproteins isolated from these fractions by extraction with alkaline ethanol (18).

The ability to replace fucose by sialic acid and vice versa as end groups of their carbohydrate may represent a fundamental structural property of glycoproteins related to their physiological functions.

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SYMPOSIUM ON BIOCHEMICAL GENETICS

Introductory remarks

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BIOCHEMICAL EVALUATION OF GENETIC PROCESSES has lately increased rather remarkably in scope and depth. One is now led to formulate the transmission of genetic information grossly as a three-step reaction through DNA and RNA to protein. On the DNA side, gene structure has been broken down to the level of relating nucleotide numbers to mutation analysis, which may be considered as a first step towards a chemistry of the gene. The chemical understanding of DNA replication, however, has become one of the most hopeful advances. The correspondence of structurally deduced and of enzymatically produced DNA-replicas has provided a first chemical expression of reproduction. You are all familiar with the successful replication of primer-DNA base composition in Kornberg's enzyme system. He mostly calls it a polymerase, but to emphasize the remarkable performance of this enzyme it deserves the name of replicase. This coupling of polymerization with replication is, understandably, in the foreground of attention.

As one proceeds from DNA to protein, a chemical understanding seems to be developing but is less clear, as yet. RNA is expected to be the next step toward the eventual expression of DNA-carried information in the protein. There are indications that specificity may be transferred from DNA to RNA, again by base pairing through hydrogen bonding. If this develops as expected, it would make the conversion DNA → DNA-replica → RNA a chemically rather homogenous reaction pattern. One might therefore consider the two reproductions of analogous base sequences as homologous replications, both expressed in the 4-base code. The translation of the genetic 4-base code message into the 20-amino acid code of proteins, being the final expression of the genetic message, obviously requires a rather complex process which should be distinguished from the two homologous ones as a heterologous replication. Here, the 4-base RNA code produces a specific amino acid sequence in an obviously gyrating manner through a not yet fully understood reaction chain. By this heterologous replication, the gene code is expressed finally as a fully developed biological system.

I have tried here to formulate some, by now, rather well-known principles, and to express them in a more descriptive manner in an attempt to recognize the biological meaning as it develops along the chemical path.

Fine structure of a genetically modified enzyme as revealed by relative affinities for modified substrate[1]

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ONE OF THE MOST SIGNIFICANT HYPOTHESES in the realm of biochemical genetics is that genes control the formation of the enzymes. There is good evidence by now that at least some genes govern at least some significant aspects of the formation of specific proteins, both enzymes and non-enzymes. Since it is also increasingly clear that the functional unit, the classical gene, possesses a 'fine structure' of many sub-units subject to independent mutational and recombinational experiences, the question has arisen whether or not, and in what way, the fine structure of mutated genes may operate to produce modified cell characters. A number of sets of modified amino acid sequences have by now been recognized in different related natural proteins, and a few of them indeed seem to be under simple genetic control (30). It is stimulating to suppose that this recognizable fine structure in the protein is a direct expression, point for point, of the recognizable fine structure of the gene material. Even this attractive conception cannot assure us that the familiar units recognized in acid hydrolysates, the amino acids and the nucleotides, are precisely the units involved in this point-for-point correspondence. Nor need such units consist unambiguously, and in an invariant manner of simple linear pairs, triads or sets of these components (24).

Alternative hypotheses might propose that only amino acid residues at certain important points—those which in the folded protein became part of an active center, for example—or those making folding possible—were specified by the 'enzyme-determining' genetic regions, while other amino acid residues were specified by cytoplasmic components, or what may be very nearly the same thing, by conjoint effects of several genes. Or, enzyme-determining genes might control the final folding or cross-linking of specific proteins, or the reversibility or irreversibility of certain steps in biosynthesis. Or again, genes may in many cases control the signal permitting or blocking enzyme synthesis, or in equivalent ways control the timing of cell functions, as has at various times been suggested (for example 20, 24, 33, 39, 44, 54).

It is a considerable task to compare peptide sequences of even two related proteins, and it is natural that few such pairs have been studied as yet. On the other hand, it is not nearly so difficult to isolate mutants and show that they are different by purely genetic test. Therefore it is not surprising that there is as yet no demonstration of the postulated control of major segments of an amino acid sequence by factors contained within a single locus in the genetic material. It may, however, justify serious concern that a number of cases of enzymes catalytically (55, 62, 63), physically (19, 23, 40) or immunologically (56, 62) modified through mutation have been recognized, yet not in general in any of those systems in which exquisite genetic fine structure has been analyzed (8, 16, 22, 47, 48). The latter seem to control essentially all-or-none functions: presence or absence of an activity, but not its modification. Without doubt, the work reported by Bonner in this symposium (10) and systems being actively studied currently in several laboratories will throw much light upon gene-enzyme relationships. The system which we shall describe in this paper illustrates the effect of changes in a moderately complex genetic fine structure upon relatively definable and limited aspects of a specific catalytic function.

We have for some time sought to define enzyme systems under genetic control in *Pneumococcus*, bacteria from which active gene material itself can be isolated as purified deoxyribonucleate (DNA) (4). Several enzymatic functions, salicin fermentation (1), mannitol dehydrogenation (43), lactate oxidation (17, 57) abnormal capsule production (5) glucose and galactose epimerase and dehydrogenase systems (2) and amylomaltase formation (38) seem to be controlled in pneumococci in an all-or-none fashion by DNA factors. Intermediate degrees of capsule synthesis (18, 41) result from what may be modification of uridinediphosphoglucose dehydrogenase activity (2), and it is possible that different forms of the heritable 'M' protein (3) antigen represent different modifications of a single protein. The *p*-aminobenzoate-utilizing system which we shall describe below was recognized as a genetically modifiable single enzyme

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in 1955 after its stepwise quantitative genetic control was first appreciated. We shall first discuss an earlier sulfonamide-resistant mutant with which preliminary evidence of a genetically modified cell function was obtained.

PARAMETERS OF MUTANT SULFONAMIDE RESISTANCE

A spontaneously arising sulfanilamide resistant pneumococcal strain, RFc, was derived in 1952 by the present authors, and used as source of DNA for certain transformations (6, 26, 42). One convenient expression for the resistance of this (and other) mutants in various media is the concentration of sulfanilamide (SA) just sufficient to cause major inhibition of growth (small colonies in antibody medium (25) even after prolonged incubation). This level is not independent of the constituents of the medium; foremost among the factors affecting growth inhibition by SA is, as might be expected the competitive metabolite, *p*-aminobenzoate (PAB). For some purposes we chose to control or buffer the low PAB content of the cells and medium by adding a known concentration of PAB. The SA inhibition level now became essentially an expression proportional to the often used molar SA/PAB ratio.

Other constituents of the medium than PAB affect the SA inhibition, however. These are probably products of the PAB-folic acid metabolism (21, 51, 60, 61) as may be pictured from the general scheme in figure 1. What we noted was that although such changes in the medium shifted the resistance level—in effect the inhibition index (51)—each such shift was the same for the wild sensitive type as for RFc and other resistant mutants. In effect, the mutant/wild type resistance ratio remained the same no matter how much inhibition was relieved by diminishing the need for folic acid. It thus appeared that the mutants were not different from wild type in their ability to produce or utilize different end products of folic acid, but probably differed in their ability to produce folic acid in the presence of sulfanilamide. The example given in table 1 was offered in 1954 (Jesup Lectures, Columbia University, unpublished) as indirect support for the hypothesis that these mutants might be resistant to SA by virtue of having altered PAB-utilizing enzymes with reduced affinities for SA relative to PAB.

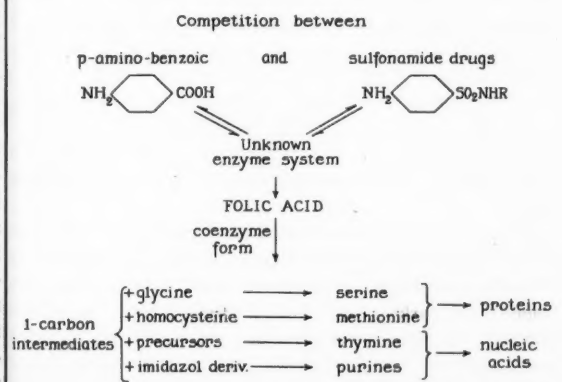

FIG. 1. Representation of relationship of sulfonamides, *p*-aminobenzoate and folic acid in bacterial metabolism.

TABLE 1. *Relative Resistance of Sulfonamide Resistant Pneumococci*

Conditions of Test	Sulfonamide Concentration for No Growth	Sulfonamide/PAB Ratio	
		No growth	Partial growth
Medium I			
wild type	10	1500	800
resistant mutant	300	32000	16000
(*Ratio:* M/WT:)	(30)	(21)	(20)
Medium II			
wild type	3	180	150
resistant mutant	100	5600	3600
(*Ratio:* M/WT:)	(33)	(31)	(24)

Sulfonamide concentration in μg sulfanilamide/ml; ratio to PAB (*p*-aminobenzoate) is molar ratio for the defined inhibition.

On the grounds that the specificity of resistance toward PAB analogs of different structure shown by a number of *Escherichia coli* mutants was incompatible with other conceivable mechanisms, Davis and Maas (15) made the explicit proposal that these mutants bore enzymes with decreased relative affinities for the analogs. We must have been considerably influenced by these suggestions, although we did not recall our early discussions with these authors until we rediscovered their interesting paper several years later. As they reported, Ivanovics (32) had previously interpreted shifts in drug/PAB ratio in resistant staphylococci to signify increased affinities of an enzyme for PAB. More generally, the concept of binding of drugs to cell 'receptors' has been applied to many drugs since the time of Ehrlich, and includes the case of sensitivity (7, 11, 35, 37) and resistance (58) toward sulfonamides. With the exception of Davis and Maas, most workers who compared sensitive with resistant strains used only one or two strains and few analogs, and so had a relatively small basis for generalization. Since in many cases also the strains were not known to be genetically related there is a strong possibility that properties unconnected with resistance may have appeared among the differences noted. In any case, the hypothesis that enzymes with genetically altered affinities were present in our RFc and similar mutants, seemed to be favored by the work mentioned above, and it is this hypothesis which we are able to support below with observations on a rather extensive related family of mutants and their specific responses to a large series of PAB analogs.

Regardless of hypothesis, the mutant/wild type resistance ratio emerges as a parameter of sulfonamide resistance more stable than other measures for cells exposed to drug in a variety of environments. It does change with pH, and it becomes a significant measure of hydrogen ion effects upon the respective cell systems, since other factors relating to drug and PAB dissociation

are changed alike. In what follows we shall make considerable use of this ratio especially to demonstrate homologies in the detailed influence of genetic composition and drug structure on resistance.

COMPLEX LOCUS DETERMINING HIGH SULFONAMIDE RESISTANCE

A new, highly sulfonamide-resistant strain of pneumococcus, RF29, was derived in 1955 from a sensitive culture by a single selective passage in drug-containing medium. Cells transformed by DNA of this strain received sulfonamide resistance determinants of three different kinds (27). A genetic analysis of RF29 indicated that the resistance locus has the genetic constitution signified by the arbitrary symbol *adb*. (28). This analysis was possible since transformation resulted in some linked transfer and some dissemination of the three determinants carried by the DNA. Transformants appear bearing the unit *a*, *d* and *b* genotypes, and also the linked multiples, *ad*, *db* and *adb*. Furthermore, the unit types can be transformed in all expected sequences by DNA of other unit types to build up the *ad*, *db* and *adb*, and in addition the *ab*, types. Repeated independent isolations indicate that these types are distinct and uniform classes.

In summary then, the genetic constitution of a DNA particle controlling sulfonamide resistance may be recognized as either *adb* (in RF29), +++ (in sensitive wild type), *a*++ (in strain Fa), *ad*+ (in strain Fad), +*d*+, +*db*, ++*b*, or *a*+*b* in the other strains.

What gives these eight strains particular interest is that they are not merely either resistant or sensitive to sulfanilamide, but show eight quantitatively distinct degrees of resistance. Representative values for resistance to sulfanilamide will be found in Table 2. If these three mutant regions, *a*, *d* and *b*, correspond to fine structure within a single gene, then the eight strains are unique in presenting so many different phenotypes, since, as mentioned, most mutations in fine structure seem to result in complete loss of a property (all-or-none behavior). And if we are dealing with a genetic fine structure, it will be all the more important to understand the nature of the phenomic alterations determined by changes in these sub-genic regions.

Although they were recognized as the result of what may have been a single mutation, it is not immediately obvious whether *a*, *d* and *b* are genes or parts of genes. Taking the gene to be that unit which is at one locus and controls either partially or totally a single cellular function, the classical gene may be virtually homologous with the 'cistron' (8). The latter can be described as that entity whose components can perform a genetic function when introduced together in the *cis* array from one parent, but generally do not function when coming to a diploid or heterozygote in the *trans* arrangement from different parents. It is not at present feasible to test pneumococcal cells to learn whether factors *a* and *d*, for example, can function together in a temporary heterozygotic or diploid arrangement. Because of their quantitatively different effects, however, it is possible to test the degree to which *a* and *d* and *b* cooperate to modify cellular functions when present together in a haploid cell in the *cis* array. It is this '*cis* test' of quantitative interaction rather than the *cis-trans* test of all-or-nothing which will indicate that they are parts of a single gene.

NATURE OF CELLULAR FUNCTIONS ALTERED BY RESISTANCE GENES

Leaning upon the fundamental observation that there is some form of correspondence between constitution of genome and enzyme composition of phenome, certain propositions are reasonable. Since mutations are events of relatively restricted extent within the genome, it may be supposed that the vast bulk of the enzyme systems of a mutant will be exactly the same as those of its parent strain. Since in transformation only similarly small fragments of a marked genome are incorporated intact into the transformant cells, these latter will in general be changed in enzymatic composition in only very limited and specific ways. This means that biochemical differences between transformant and parental strains will almost certainly be referable to the specific change selected in isolating the transformant. By combining the sulfonamide resistance factors *a*, *d* and *b* in the different multiple strains mentioned above, we are able to compare the effect of each genic factor against several genetic backgrounds.

The general finding in such comparisons is that the factors can substitute in part for each other and when combined can powerfully potentiate each other. Thus, either *a* or *d* or *b* mutation, alone, can make growth possible beyond the usual inhibitory concentration of sulfanilamide, 5 μg/ml, so each unit must in some way operate to give the same end products necessary for growth. Furthermore, any combination of two or more of these genic factors gives still greater resistance than its component factors alone. Growth inhibition by SA for any combination is specifically relieved by PAB, and when there is growth inhibition it is correlated with suppression of folic acid synthesis. The only explanation of these observations that seems acceptable is that the factors *a*, *d* and *b* all modify in different ways the quantitative behavior of the same enzyme, one which utilizes PAB and leads to the formation of folic acid. A systematic presentation of the reasoning involved, together with some of the supporting data, is given in detail in table 2.

Cis-test for gene interaction. The important indication of multiple modifications in a single enzyme came from the enhanced effect due to pairs of the markers. Whatever entity is modified by one marker would have to be further changed by the second marker in order to function adequately at a considerably higher level of SA (conclusion 5, table 2). We have called this the '*cis*-test' for interaction since with two quantitative markers that can

TABLE 2. *Identification of Three Genetically Altered Functions Within a Single Enzyme*

Hypothesis. Resistance mutations *a*, *d* and *b* result in three enzymatic alterations (A, D and B becoming respectively AR, DR and BR)—either in three enzymes functioning sequentially in one system, or independently in parallel systems, or in three aspects of a single enzyme.

Strain	State of Enzymes in Strain				Half Normal Growth		50% Folic Synthesis
	1	2	3	All others	SA level μg/ml	SA/PAB ratio	SA μg/ml
wild	A	D	B	resistant	5	450	2·5
Fa	AR	D	B	resistant	20	1800	7·5
Fd	A	DR	B	resistant	80	7200	40
Fb	A	D	BR	resistant	15	1300	6

Properties common to all three enzyme

Observation

1. Each enzyme when it undergoes change is able to release growth to new higher levels of SA.
2. Mutation to *a*, giving AR (altered A) enzyme, releases growth to 20 μg SA (and BR in *b* to 15). Yet strain *d*, with normal A, having DR can grow at 80 μg SA.
3. Growth inhibition is competitively released by PAB for all strains up to saturation of solution with SA.
4. Each growth inhibition effect is roughly paralleled by inhibitory effect upon folic acid synthesis.

Conclusion

1. The product of each enzyme is necessary for growth. Each enzyme can be inhibited by SA.
2. Enzyme A (or B) cannot be functioning adequately in strain *d* at 80 μg SA. Therefore the enzymes D, A and B must contribute to make the same end products, but cannot be sequential.
3. PAB is a primary participant in all three enzyme systems. No other enzyme system important for growth is affected by SA.
4. The eventual common product of all three enzyme systems is folic acid.

Summary. The three functions may be different enzymes or parts of the same one, but they operate in parallel to utilize and produce products convertible into folic acid.

The 'cis' test for interaction of genes having quantitative effects

Principle. When two quantitative markers are superimposed in *cis* configuration in a haploid cell, their mode of addition can reveal any interrelation in their phenotypic effects. Thus, if two drug resistances are superimposed, if they are unconnected, the aggregate may be no more resistant than the lesser one, and should never be more so than the more resistant one.

Strain	State of Enzymes in Strain				Half Normal Growth		Relative Resistance
	1	2	3	All others	SA level μg/ml	SA/PAB ratio	
Fad	AR	DR	B	resistant	400	35,000	80
Fdb	A	DR	BR	resistant	300	27,000	60
Fab	AR	D	BR	resistant	70	6,400	14
Fadb	AR	DR	BR	resistant	1200	110,000	240

Observation

5. When marker *a* is combined with *d*, the doubly marked Fad strain can grow in 400 μg SA which inhibits Fa and Fd. Similarly, Fdb and the other multiples have high resistance.
6. DNA of mutant *a* (with 4 × resistance relative to wild type) acts upon genotype *d* (16 ×) to make *ad* (80 ×), likewise with *b* (3 ×) it gives *ab* (14 ×) and with *db* (60 ×) it gives *adb* (240 ×).
7. Similarly, *b* and *d* each make their own characteristic addition to resistance in each of the four strains which lack them.
8. Mutant/wild type resistance ratios are the reciprocal of sensitivity ratios for the same drug.
9. Similar essentially factorial additivity is obtained for sensitivity of all markers toward a large number of other PAB analogs.

Conclusion

5. The mutation *a* (and also *b*) affects the DR enzyme as well as the A; or *d* affects the AR enzyme. Therefore, A and D and B are the same enzyme.
6. Marker *a* makes virtually the same change in the common enzyme when added to each of the four strains, wild, *d*, *b* and *db*.
7. All three marker regions act independently to control quantitative properties of a single enzyme which reacts with PAB and is inhibited by sulfonamides.
8. Sensitivity, or relative affinity of drug for enzyme surface is also additive factorially.
9. The three mutant regions *a*, *d* and *b* have virtually independent characteristic effects upon the relative affinities of an enzyme site for drug and PAB.

be evaluated in separate strains (the analog of the *trans* configuration) one can predict certain types of additivity that may occur when the markers are together in the *cis* arrangement. By contrast, with markers that result in total enzyme deficiency, or markers modifying such a qualitative trait as coloration, one normally will not know what either independent or interacting addition of their effects might give. In these cases one must contrive to test a *trans* heterozygote or heterokaryon as well, to complete the usual test for cistronic relationship. In the case of the pneumococcal mutants which recombine to produce different amounts of capsular antigen (18, 50), the antigen itself is not a primary gene product. Therefore, although the determinants seem to be linked (50), any estimate of their cistronic relationship will depend upon how uniquely their

effects can be recognized upon the two or more enzymes that are probably more direct agents of the modified capsule synthesis (2).

MODIFIED ENZYME UTILIZING *p*-AMINOBENZOATE

Evidence will be presented below that many different analogs of PAB show markedly different specificities of inhibition toward the Fa-d-b series of mutants. These numerous observations rule out alternative explanations of resistance (15) based on increased accumulation of PAB or metabolic changes obviating or sparing the need for PAB. It is also unlikely that resistance can result from metabolic conversion or catalyzed accumulation of such a wide variety of compounds. As will be discussed at some length later, their inhibitory ability as analogs of PAB seem to rest upon the electron distribution of their substituent groups, and is not in keeping with their ability to undergo analogous chemical reactions.

The modified enzyme catalyzing the reaction

$$PAB + X \rightarrow PABX$$

and responding to sulfonamide analogs in the various mutants should have those features indicated above and others to be described below, whatever part it plays in converting PAB into folic acid. If it is an enzyme accomplishing active transport of PAB and analogs into the cells (a 'permease'), the component X in the reaction above may be thought of as (cells) and PABX as PAB (internal). The competitive effect of drug concentration shows that any such enzyme would have to concentrate PAB, or SA, etc., reversibly and selectively in proportion to their external concentrations. Otherwise growth would be merely slowed until $PAB_{internal}$ was built up to an optimal level. Or else one would have to assume that both the concentrating enzyme and also an internal PAB-converting enzyme were subject to the competitive inhibition. Another possibility might be that PAB is freely permeable and each analog is enzymatically concentrated to different extents into the different mutant cells, where it has equal inhibitory effect. One can invent one or two similar hypotheses for each diffusion boundary or interacting metabolite (for each X) one is willing to assume. All of these hypotheses have one feature in common, a genetically altered specific cellular component.

More direct evidence has been obtained indicating that the Fa-d-b mutants do not differ in their rate of production or normal utilization of PAB. A method for estimating the endogenous PAB levels in growing cells is presented in table 3. The method uses the added drug concentration needed to balance an increased PAB concentration to give an extrapolated estimate of the PAB contributed by the cells. It should be pointed out that this method: *1*) measures all metabolites competitively antagonizing sulfonamide inhibition, *2*) gives its results in terms of an external concentration of PAB equivalent in effect to the endogenously produced metabolite, and *3*) measures endogenous metabolite in the steady state existing at the chosen level of growth inhibition.

TABLE 3. *Endogenous PAB Determination in Pneumococci*

pH	Endogenous PAB, mμg/ml, in strain:							
	wild	*a*	*d*	*b*	*ad*	*db*	*ab*	*adb*
7.5	14	12	14	11	18	12	11	10
6.5	3	4	2	2	2.7	1.5	3.8	4.5

Principle. 1) Point of defined inhibition will occur when external drug concentration, I_0, reaches a fixed ratio to the endogenous PAB concentration, P_0, equal to I_0/P_0, characteristic for the strain and conditions. *2*) For an added PAB concentration, P_1, an additional concentration of drug, I_1, will be required. *3*) The new ratio, $I_1/(P_0 + P_1)$ will be equal to I_0/P_0. *4*) Effective endogenous steady state concentration of PAB is hen: $P_0 = (P_1 I_0)/(I_1 - I_0)$. *Application.* All 8 mutants show the same endogenous PAB in meat infusion-neopeptone medium, and show similar change with pH.

P_0 values obtained with SA, sulfanilate, diaminodiphenylsulfone, nitrobenzoate, *p*-aminosalicylate, are alike within experimental error. This series includes ionizing and not-ionizing sulfones and carboxylates, with inhibitory ratios, I_0/P_0, covering a 100-fold range.

PAB, mμg/ml:	0	20	40	80	160	320	640
Drug, μg/ml:	0.19	0.43	1.0	1.4	2.5	5.5	11
P_0, mμg/ml:	—	15	9.5	12	13	11	11

Drug used in the last experiment: *p-p'*-diaminodiphenylsulfone. P_0 values remain constant over a 60-fold range of drug.

So measured, the steady-state PAB level of each of the eight mutants is found to be the same within experimental error (see table 3). The same endogenous PAB concentration, is found using one drug over a 60-fold range of concentration, or using two drugs of widely different inhibitory activity or different acid dissociation. Changes in the medium can affect this parameter, a change of pH being notably effective; a drop from pH 7.5 to 6.5 in neopeptone broth lowers the endogenous PAB from 0.012 (± 0.002) μg/ml to 0.003 (± 0.001) μg/ml equivalent concentration. Again, all of our eight strains (and others) demonstrated their homogeneity by showing the same shifts in PAB synthesis with acidification of the medium. These facts indicate that the resistance mutations are not correlated with any change in production or ability to concentrate PAB, nor probably with an altered need or capacity for utilizing it.

It has been mentioned that synthesis of folic acid is inhibited in parallel with growth when the different mutants are exposed to various analogs. 'Folic acid' as defined by bioassay is in reality a series of closely related substances, differing in presence or absence of glutamyl residues, formyl groups, and hydrogenation of the pteridine moiety. For our immediate purpose it is satisfactory that bioassay with *Streptococcus fecalis* measures many of these substances (49), and the autogenous bioassay with pneumococcus itself (to be published) which we chiefly use is even more suitable for measuring pneumococcal synthesis. Not all of the inhibitors to be

presented below have been tested for their effect upon synthesis of the folic acids, but for all those which have been tested, the general level of sensitivity and mutant specificity are the same whether colony growth or folic acid biosynthesis is measured.

A more direct test of inhibition mechanisms will be available when the steps in synthesis of folic acid from PAB are clarified, and enzyme preparations become available. We are investigating folic acid synthesis in cell-free preparations from pneumococci. Synthesis thus far observed is slight, perhaps because a number of enzymatic steps are involved and some of them have been severely disturbed when the cells are disrupted. It seemed that it would be desirable to measure a product more closely related than folic acid to the first PAB-utilizing reaction. Attempts to identify an early intermediate (PABX above) have so far given equivocal results. With Dr. C. DeLuca in this laboratory we have isolated metabolites produced from PAB and *p*-aminosalicylate by two different strains. These labile substances were recovered in only small amounts and though they appeared to liberate the free *p*-amino acid on hydrolysis, we could not demonstrate any conversion to folic acid when they were added back to whole cells. Our metabolites differed from those produced by *Escherichia coli* (31) or from those recovered from mycobacteria by Sloane (53) and generously supplied by him for comparison. We are also studying the formation of folic acid by washed cells, somewhat along the lines of early work in Woods' laboratory (45). The mutants show here also the pattern of specific sensitivities predicted from growth inhibition.

In recent years, several reports on mechanism of biosynthesis of folic acids have appeared, but the results in different species are somewhat different. In mycobacterial enzyme preparations, *p*-aminobenzoylglutamate seems to be the active intermediate (34). Extracts of lactobacilli were able to couple pteridines to PAB whether or not it was first coupled to glutamate (52); *Escherichia coli* gave active extracts which show similar behavior (12). At the present, we cannot yet say whether PAB normally combines first with glutamate or with pteridine or pteridine precursors,[3] (nor of course whether active derivatives with pyrophosphate, adenylate or Coenzyme A may be involved) in pneumococcus. We can say that the mutants discussed here synthesize typical folic compounds and by means of enzyme systems which are fundamentally responsible for the specific genetically modified drug sensitivity patterns. In what follows we shall attempt to identify fairly intimate properties of the reactive enzyme surface. In some senses this can be a more profound description than a simple designation of the over-all reaction which it catalyzes—a tentative designation, since the reaction would in all likelihood prove later to involve one or two extra intermediate reaction stages.

[3] The simple test as to whether pteroic acid or *p*-aminobenzoylglutamate can be utilized non-competitively is inconclusive as neither substance is used efficiently. The question is probably complicated by low permeability for these substances and the possibility that the reduced pteridines are the more natural forms.

ANALYSIS OF ENZYMATIC FINE STRUCTURE BY SUBSTRATE INHIBITION STUDIES

The eight different forms of the PAB-utilizing enzyme present in the eight strains of the Fa-d-b family have already demonstrated their quantitatively different responses to the inhibitor sulfanilamide, SA. The concentration of SA needed to produce a certain reduction in growth can be taken as a measure of the amount needed to reduce to a certain fraction the number of enzyme sites available for PAB conversion. That would make this concentration an inverse function of the affinity of the drug for the enzyme; or the drug/PAB ratio should be directly proportional to the relative PAB/drug affinities for the enzyme. Any effect of permeability differences should, as outlined above, merely change the proportionality constant, unless as seems unlikely the permeability system is the one which is mutationally altered, and then it would become the system which we are in effect analyzing.

The most noticeable effect of changing from SA to another drug of different structure is that a different absolute level of sensitivity is found for the strains. Thus, wild type pneumococcus is 12 to 15 times more resistant to SA than to sulfathiazole. A more useful way to state such comparisons here is in terms of reactivity, thus, the wild strain is 12 to 15 times more reactive, or sensitive, to sulfathiazole than to SA. Similar differences are found for many of the common sulfonamides, for example our wild strain is approximately as sensitive to sulfadiazine as to SA, 8 to 10 times more sensitive to sulfapyridine, but only $\frac{1}{15}$th to $\frac{1}{20}$th as sensitive to sulfacetamide or sulfanilic acid.

Role of ionization in drug reactivity. The differences mentioned fit in a general way with the theoretical formulations which postulate a relationship between antibacterial activity of sulfonamides and an optimal acid dissociation constant (7, 14, 35). Thus, the substances of pKa in the range 7 to 8 (sulfathiazole and sulfapyridine) are highly active, those of somewhat greater (sulfadiazine) or lower (SA) acidity are less reactive, while the most acidic (sulfacetamide and sulfanilic acid) are of low activity.

Nevertheless, the theories mentioned were principally designed with the N^1-substituted sulfanilamides (of chemotherapeutic interest) in mind, and do not help us to predict the reactivity of compounds of slightly different nature. One finds that ability to ionize is not essential for activity, for both sulfaguanidine and *p-p'*-diaminodiphenylsulfone are highly effective competitors of PAB, yet they do not or cannot dissociate a proton from their sulfone groups. Again, although the weakly active sulfanilic acid is more acidic than the optimum postulated by Bell and Roblin (7), its low activity cannot be attributed to electron withdrawal from the sulfone oxygen atoms, since there is no substit-

TABLE 4. *Relative Resistance of Unit Mutant Strains to Various Sulfonamides*

Strain	SA	ST	SD	SP	SG	SAC	ADS	ATS	AES
	Absolute inhibitory concentration (μg/ml)								
Wild	5	0.25	3	0.5	1	16	0.2	20	50
	Relative resistance: mutant/wild type								
Wild	1	1	1	1	1	1	1	1	1
a	4	5	2.7	4	6	4	6.5	1.5	3
d	16	12	8	10	20	10	13	6	6
b	3	1.6	1.7	2	3	2	3.5	1.8	3

Drugs used: SA, sulfanilamide; ST, sulfathiazole; SD, sulfadiazine; SP, sulfapyridine; SG, sulfaguanidine; SAC, sulfacetamide; and the sulfones: ADS, *p-p'*-diaminodiphenylsulfone; ATS, 2-sulfanilylthiazole; AES, 4-aminophenyl-(2'-diethylaminoethyl)-sulfone.

uent group on the sulfonate group. On the other hand, it might be maintained that the sulfanilate is nevertheless somewhat more active than an equally acidic sulfonamide would be.

Whatever regularities one can find for the reactivity of one strain of bacteria toward different drugs do, however, tend to disappear when different bacteria are tested. It is fairly certain that a consistent ionization theory could hardly have been constructed explaining antibacterial activities toward more than one organism (*E. coli* was used in the work cited). Our wild strain of pneumococcus is 10 times more sensitive than *E. coli* (7) to sulfaguanidine and $\frac{1}{40}$ as sensitive to sulfadiazine (both relative to sulfapyridine which is of similar activity).[4] We shall see that genetic differences between even closely related mutants can be of decisive importance in determining sensitivities to various drugs of similar structure.

However, first comparisons of the basic unit strains showed that *d* was always most resistant, and the pattern was much the same for all sulfonamides. The order of increasing sensitivity was always $d < a < b <$ wild,[5] with *d* usually $\frac{1}{10}$ to $\frac{1}{20}$ as reactive as wild type strain, and $\frac{1}{4}$ to $\frac{2}{5}$ as reactive as *a*, the next most resistant. Data are presented in terms of mutant/wild type ratios: in table 4 and tables to follow, as resistance ratios since these are convenient integral numbers and are directly useful in devising selective media. In discussion we shall usually deal with the reciprocal, the mutant/wild type sensitivity ratio.

There are specific and reproducible differences between the sulfonamides, but the pattern mentioned seems to dominate. An early sign that this pattern would not hold for all analogs of PAB however, was the response obtained with *p*-aminosalicylate, to be discussed more at length later. With the latter substance, the sensitivity order was $b < a < d =$ wild. It seemed that a different feature was present in this compound, toward which the *b* mutant was least reactive and the *d* most, whereas for sulfonamides the reactivity was in the converse order. For example, it was the weakly ionizing sulfonamide class which reacted poorly with the *d* enzyme and more with the *b*, while the highly ionized aminosalicylate reacted well with the *d* and poorly with the *b*. We thought at this point that, superimposed upon an absolute scale of differences in affinity for the various sulfonamides, affinities more or less correlated with their ionization but not dependent upon it, there was a predominating affinity of the *d* enzyme, greater than others, for an ionized group (carboxylate or sulfamyl ion). Our early formulation (28) was that the *d* enzyme, having an affinity for carboxylates, reacted with PAB to the relative exclusion of sulfonamides, but could not so well exclude the carboxylate analogs.

In principle, we are now trying to discern the effect of chemical structure on inhibitory activity by observing in what refined ways the activities can differ between mutants (in which other biochemical properties are alike), rather than trying to understand inhibition of a single strain without any standard for comparison other than our own a priori conceptions of what is different about two compounds. Comparison of strain responses reminds us that acidity alone as the single basis of affinity cannot explain why wild type is most reactive with both types of analog. Nor is it in the least supported by the data for other sulfone-containing drugs (table 4). The nonionizing sulfaguanidine and *p-p'*-diaminodiphenylsulfone are highly active and the completely ionizing sulfanilate rather weakly so. Yet for each the 'sulfone type' of relationship is found, sensitivities on the pattern $d < a < b <$ wild, with ratios quantitatively similar to those of the average sulfone, 1:2.5:5:13. Only moderately exceptional is the sulfanilate, which reacts with *d* almost as well as with *a*, but still at the lowest level of responsiveness. Other sulfones which do not bear two symmetrical *p*-aminophenyl groups are less reactive but still typical members of the group.

Thus, the pattern $d < a < b <$ wild for reactivity persists over a range of sulfone-containing drugs varying in absolute activity by 100-fold and from unionizing to highly ionized acids. Whatever renders *d* enzyme unreactive and the others successively to wild type more

[4] The experimental conditions have been described (28) and briefly are as follows: *Inoculum:* 10 to 100 cells of stock genetically marked strains from cultures recently grown to maximum turbidity in neopeptone-meat infusion medium. *Test broth*—above medium diluted 1:3 with saline, pH about 7.4, containing agglutinating antibody (25), in 1 cc quantities containing accurate dilutions of neutralized drug. Dilution steps about 1:3 in preliminary work, 1:1.5 in ultimate work. *Observation*—index concentration is that in which colonies grow to about $\frac{1}{4}$ maximum diameter on long incubation. Interpolation made on basis of comparable other series if colonies are too big or too small in levels chosen: The pH of these tests is slightly higher than 7 which was chosen by Bell and Roblin but not in such a way as to explain the quite different relative sensitivities mentioned.

[5] For simplicity in this section we shall refer to standard strains, not by actual strain number, or type (Fa, Fb, etc.), but by genotype designation: *a*, *d*, *ad*, etc. The data represent in most cases tests on more than one strain, sometimes many, which behaved essentially alike. They include for almost all compounds tests on one selected set of single colony strains, set 7, with strain numbers like RF2-7, RF4-7, RF6-7 and RF26-7, (representing *b*, *a*, *d* and *db* respectively).

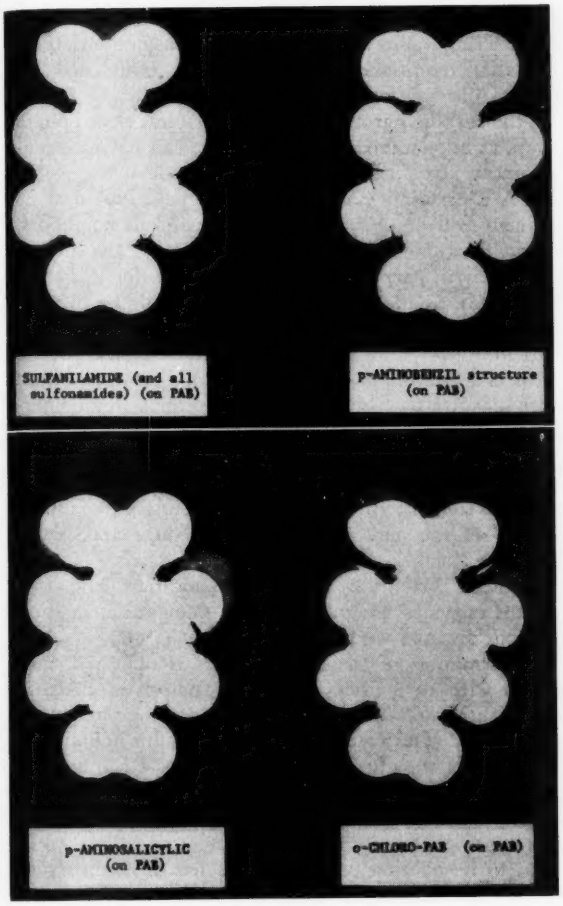

FIG. 2. Shadowgrams for molecular models, representing the spatial relationship of superimposed pairs of compounds. The differences (half-shadow) indicate the space requirements of the various compounds in excess of those for *p*-aminobenzoate (PAB, shown as completely white).

reactive, would seem to relate less to ionization than to other features of the sulfone structure which contrast with those of the carboxylate. Among these we should consider the space-filling properties of the rather large sulfone group, possible intrinsic spatial or electronic properties of the sulfur atom itself, and the electron density about the sulfone oxygen atoms. The region of these oxygen atoms should be quite electron-rich, since it is probably affected by sharing in resonance the π-electrons of the benzene ring. Most of these aspects were carefully considered by Bell and Roblin as fundamental to analogy with PAB, before they developed the view that ionization was not merely the measure, but also the basis of ability to combine with *E. coli* enzyme (7). Kumler and coworkers have maintained that resonance hybrids are the reactive form of PAB and its analogs (37), but stress the resonance form (quinoid, positive nitrogen) of the *para* amino group even more than the negative sulfone oxygen atoms.

TABLE 5. *Relative Resistance of Strains to Drugs With Modified Carboxyl*

Strain	Nature of Chemical Group Replacing Carboxyl					
	sulfone	sulfonate	benzil	acetyl	nitro	cyano
	Absolute inhibitory concentration (μg/ml)					
Wild	16	15	1.6	225	100	50
	Relative resistance: mutant/wild type					
Wild	1	1	1	1	1	1
a	4	5	1.2	2.2	3	3
d	10	5	12	1.3	3	2.5
b	2	1.7	2.2	1.8	2.5	2.6
ad	43	16	55	6	7	10
db	30	10	100	9	8	6
ab	6	5	24	7	3.5	6
adb	75	50	125	12	10	24

Drugs used: sulfone, sulfacetamide; sulfonate, sulfanilate; benzil, *p*-*p*′-diaminobenzil; acetyl, *p*-aminoacetophenone; nitro, *p*-nitraniline; cyano, *p*-aminobenzonitrile.

Since there are few quantitative predictions, on few active compounds, to prove or disprove their hypotheses, it seems best to seek, using similar principles, a qualitatively satisfactory picture which fits a larger amount of observation. In this respect our family of resistant mutants and the large series of compounds we have tested provide a wealth of comparisons which at once render the feasible hypotheses less numerous, more sober, and, one may hope, more realistic.

Drugs intermediate between the sulfones and carboxylates. If the sulfones (including the sulfonamides) differ from the carboxylates by several features as mentioned above, one may find structures intermediate between the two types. Since ionization did not seem to provide a trustworthy parallel with activity,[6] we attempted to separate the contributions of electron density and steric effects.

In *p*-*p*′-diaminobenzil the dicarbonyl or α-diketone structure is of approximately the same bulk as the sulfone group. Shadowgrams of both are shown in figure 2, as half shadows (of molecular models) superimposed upon the PAB structure. The second benzene ring of diphenylsulfones or benzil, and the substituted amide, -NH-R, of sulfonamides are almost certainly out of the plane of the other ring, and they are not represented in the shadowgrams. Since the size of the N^1-substituents of sulfonamides has never shown much bearing on biological activity, it is probable that this group does not very closely approach the enzyme surface either. If resonance between the two rings and the oxygen atoms is sufficient, the two rings of the diphenylsulfones and benzil may tend to be brought into one plane. In any case, these diamino compounds have a symmetry that may contribute to easier fit and higher activity.

Most significant seems the fact that the —CO—CO— of the benzil derivative is similar in space requirements

[6] The ionization of the PAB carboxylate group might be irrelevant for binding to enzyme if while attached to it the enzyme serves to couple it to glutamic acid and forms the unionized benzoyl amide.

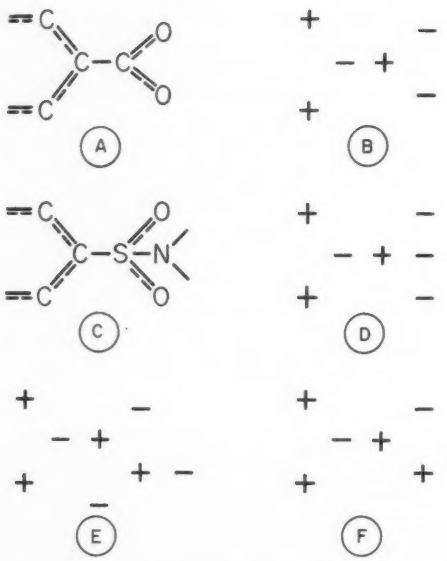

FIG. 3. Schematic diagram of electronic patterns of: A and B—*p*-amino- (and other) benzoic acids; B—nitro (aniline or benzoate) compounds; C—sulfanilamides; D—sulfonamides, sulfanilate and sulfones; E—diaminobenzil; F—(amino or nitro) acetophenones. The negative and positive charges do not represent fixed charges residing at the corresponding atomic positions. They are not quantitative but qualitative expressions of the charges which the atoms may, (at different times) assume. To illustrate, the negative charges at the right of D represent and include to different degrees all of these: *a*) electron density about the oxygen atoms of the $—SO_2—$ or sulfanilic acid group, or the nitrogen of unionized sulfonamides, drawn from the neighboring atoms, *b*) resonance electrons shared by the sulfone oxygens (and possibly amide nitrogen) with the carbons of the benzene ring, *c*) net negative charge of the sulfamyl or sulfanilate ions. The resultant negativity of carbons in the benzene ring is also suggested.

to the sulfones (fig. 2), and that the strains respond to it as they do to sulfones: $d < a < b <$ wild (table 5). There is no intrinsic specificity of the sulfur atom for binding, as may have been clear since the active benzil compounds were introduced (36). The electron distribution about the dicarbonyl group, symmetrically affected by the aromatic rings, must be somewhat different from that of the sulfone compounds. An attempt is made in figure 3 to show both the spatial distribution and the charge density pattern in a kind of summary of all of the electron displacements and accumulations that may, perhaps at different times, be represented in these structures. The result is, or could be, a picture of the structural features which are most pertinent to molecular interactions. Whatever weaknesses there may be in the assumptions used, the sulfone pattern C and D differ from the PAB pattern A and B both in size and in electron field configuration. Since the benzil structure, E, is even more different in electron distribution, we conclude that their similar relative behavior toward the mutants is in large degree the result of their space requirements. That is, the *d* enzyme is more strictly limited than the others in its ability to react with bulky analogs of the carboxylate group. The *a* enzyme and the others in the series would seem to have diminishing strictness of spatial requirement, possibly more influenced by electron distribution.

If the carbonyl group is adjacent to a less electrophilic group, as in *p*-aminoacetophenone, we can have a structure more like F. The $—CH_3$ component of the —CO $—CH_3$ is certainly not very strongly polarized, but its tendency will be to surrender electrons to its neighbors, so that the (unionizable) hydrogen atoms of the methyl group may in part justify representation E for this compound also. Spatially the group is rather bulky but not rigid, and this is reflected in a low order of activity as PAB inhibitor. But, as denoted in table 5, the special status of *d* enzyme does not appear; *d* is now somewhat more sensitive than the other unit mutants. Remembering that the special status had to a degree also been reduced for sulfanilate, the smallest of the sulfone structures, we are now prepared to conclude that the *d* enzyme has steric requirements partly excluding the bulkier analogs of the carboxylate central group, and partly operating against the bulky —NH—R or $—C_6H_4X$ substituents which they may bear in addition. The other mutant enzymes are relatively less demanding in these respects, and the wild type least of all.

Confirmation of these conclusions is obtained from results with two other inhibitory analogs of PAB, in which nitro and nitrile groups occupy the site of the carboxylate. The first of these, *p*-nitraniline (configuration B) is of very nearly the same size as PAB, and the second is rather smaller. As will be seen from table 5, all relative advantage of the *d* mutants over the other unit types disappears for these compounds. This would seem to be all that could be expected in confirmation of the steric limitations imposed by the *d* mutation.

It should be noted that reactivity in the sense of ability to undergo equivalent chemical reactions is very different indeed for such different groups as: $—SO_3^-$, $—SO_2—R$, $—SO_2—NH—R$, —CO—CO—, $—CO—CH_3$, $—NO_2$ and —CN. Consequently, we believe we are justified in considering only the ability to fit, and complex with, complementary molecular groupings. Also, it seems unlikely that the complementary structure, modifiable by fine genetic mutations, is that of an enzyme engaged in active transportation of such diverse molecules into the cell.

The additivity of the single genetic influences will be discussed later, but is revealed in the table. At this point, it may be pointed out that the influences are independent enough to add more or less factorially in every case.

Analogs of PAB with modified amino group. It was noted early in the practical development of sulfonamides that very few compounds with a modification of the 4-amino group were inhibitory for bacteria. Some of the force of this generalization disappears as one moves away from the sulfonamides, not attempting to modify both the amino and the carboxyl at once,[7] that is, introduces a

[7] Strictly speaking, every modification in one part of the PAB molecule may affect the resonance and electron distribution for

para substituent into benzoic acid. *p*-Nitrobenzoic acid is known to be inhibitory; it is believed to inhibit both a PAB system and a *p*-hydroxybenzoate system in *E. coli* (15). In our pneumococci the latter relationship does not seem to exist, but *p*-nitrobenzoate is rather strongly inhibitory and is completely reversed competitively by PAB.

When the mutants were investigated, we were surprised to find that the *d* mutants were strongly inhibited by the nitrobenzoate, being about 40 times as reactive as the wild strain. Strikingly enough, the very mutation or transformation which had given the most resistance toward all of the sulfonamides had introduced a pronounced sensitivity to a different substance. All transformants behaved alike; the one trait has never been found separately from the other.

The change from the $-NH_2$ to $-NO_2$ is rather extreme; the amino nitrogen is relatively electron-rich while the nitrogen attached to oxygen atoms will be electron poor with respect to its neighbors. It is surprising that the nitro compound serves as inhibitory analog of the amino compound, and suggests that these groups may not be important for combination with the enzyme. Or it may be that their steric configuration, which is quite similar, is more important than their charge distribution. It is not likely that reduction of the nitro compound to another form brings about its activity toward the bacteria, for late, heavy cultures of pneumococcus reduce it only slowly, yet its inhibitory action, strictly concentration-dependent, can be demonstrated in a few minutes upon cells synthesizing folic acid.

Could it be that the *d* enzyme has specific affinity for the $- + -$ spacing of the nitro group (B, fig. 3)? This can hardly be true, since a very similar pattern if inhibition is found when the *p* hydroxy group, or the *p*-chloro atom, are substituted in its place. These are electron-supplying negative groups like the amino; their effects upon ionization—of the very *para*-substituted benzoic acids themselves—ranges from suppression (amino, hydroxyl), to weak enhancement (chloro), to strong enhancement (nitro). Acetylaminobenzoic acid, as yet incompletely investigated, seems also to affect the *d* enzyme. When the aminoacetophenone of table 5, toward which the *d* was found to be only slightly resistant, is compared with the nitroacetophenone (PNA in table 6), it is the *d* enzyme again which is relatively the most reactive with the nitro form.

It seems to be clear that the *d* enzyme, most specific in its space requirements about position 1 of PAB, is least strict with respect to reaction with several rather different groups occupying position 4. It would appear that the affinities expressed toward the 4-position are undemanding of charge specificity, possibly also in

other groups especially those in para relationship to each other upon the ring. However, those effects may be considered of second order importance when the closely similar analogs of the carboxyl group are compared. And since rather profound changes in the substituent at the para-amino site have in certain cases little effect, we shall limit our discussion mainly to the effect of changes upon the site changed.

TABLE 6. *Relative Resistance Toward Drugs With Modified Amino Group*

Strain	Nature of Group Replacing Amino Group				
	chloro	nitro	hydroxyl	acetamino	(PNA)
	Absolute inhibitory concentration (μg/ml)				
Wild	1200	20	1200	ca. 800	200
	Relative resistance: mutant/wild type				
Wild	1	1	1	1	1
a	—	3	>3	—	1.7
d	0.4	0.03	0.02	<0.2	0.7
b	0.8	3	2	<0.5	2
ad	>1	0.08	0.15	—	—
db	0.8	0.07	0.10	—	—
ab	0.8	4	>3	—	2
adb	—	0.1	0.25	—	3

Drugs used: the following *para*-substituted benzoic acids: chloro-, nitro-, hydroxy- and acetamino-; PNA is not a benzoic acid, but the related compound, *p*-nitroacetophenone.

spatial requirements. For enzymes of *a* and *b* configuration, virtually every change in either the amino or carboxyl structure moderately reduces the tendency to react. It is now time to attempt to discover in what respects the traits we have seemed to find in the unit marker strains are intrinsic and fundamental.

Identification of unit traits in multiply marked strains. In the analysis thus far, the procedure has been to confirm each suspected trait of a strain by exposing cells of that strain to different drugs of nearly similar structure. Those specific affinities which have been so detected have emerged because they seemed significant in determining the behavior of the cells in response to a number of related drugs. It will be remembered that these relative affinities for drug and PAB remain unchanged when cells are exposed to them in media which spare in different degrees the folic acid requirement. Let us now consider how these selective traits survive when developed against different genetic backgrounds. Some facts have already been presented but not discussed.

An important argument for the single enzyme undergoing multiple modifications was the interrelation of the resistance traits (toward SA); that is, their ability powerfully to modify each other. It did not matter for this argument exactly how much the one resistance enhanced the other so long as it was a substantial amount. The relatively large numbers representing the relative resistances of the multiple strains in Tables 4 and 5 amply illustrate that virtually every drug we have tested reveals considerable cumulative effect of combinations of the unit traits.

It should be pointed out that the known genetic background allows us to retest each hypothesis in several ways. If *d*, for example, is found to be different from wild type in having a certain pattern of response to one class of structural changes, we can compare the wild-to-*d* difference in three other ways: *a*-to-*ad*, *b*-to-*db*, and *ab*-to-*adb*. The same is true for every other unit marker.

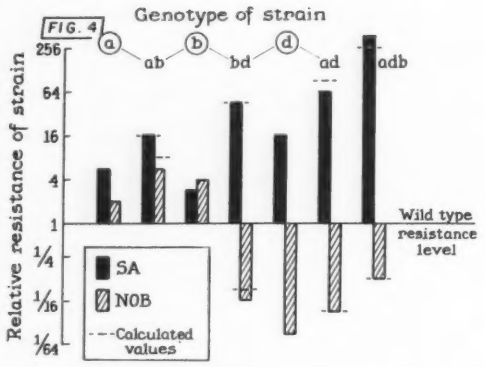

FIG. 4. The resistance levels for sulfanilamide (SA) and *p*-nitrobenzoate (NOB), determined in a single experiment are represented as ratios to wild type resistance. On the logarithmic scale are also shown (dotted lines) the resistance level calculated from the single resistance factors. The conditions of the experiment (high pH, large inoculum, short incubations, different medium) account for the independent results, slightly different from those of tables 2, 4, and 6.

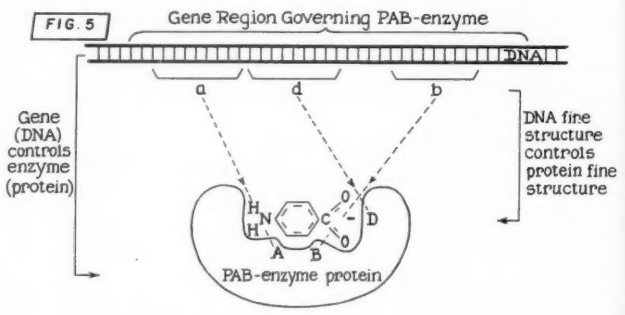

FIG. 5. Scheme illustrating relations of genes and their parts to corresponding parts of a cellular enzyme.

What we are saying is that every qualitative claim we shall make has already passed these confirmatory tests.

If the unit markers act cooperatively, yet independently, they provide additional ways to judge the nature of their cooperation. The additivity for SA is indicated in table 2 and in figure 4 it is shown on a logarithmic scale as a function of wild type resistance. It is easily seen that the resistances not only are multiplicative, but the resistance factors for each marker are accumulated almost quantitatively in the multiple strains. The same can be demonstrated for all of the sulfone drugs tested.

The *d* form of the enzyme has been inferred to show severe restriction, mainly steric, upon reactions with substances having bulky constituents at position 1 (carboxylate); *a* and *b* forms show lesser restriction. If these restrictions were all exerted in essentially the same ways (for example by changes in the amino acid residues so that the space on the active enzyme surface became narrower), then it might not be possible to enhance materially the resistance of the *d* by making it an *ad*. If the *a* and *b* enzymes exerted a selectivity toward the drugs principally based upon charge fields, however, they could add significant resistance to the *d* and still be independent of each other. The general low resistance of both *a* and *b* toward almost all modifications at positions 1 and 4 would be in keeping with this possibility. Since a part of the *d* selectivity seems to be expressed against bulky terminal groups beyond, or distal to, the sulfone, there is also a possibility that either *a* or *b* might have unique steric requirements directed more toward the proximal aspect of the sulfone group. But it does not seem likely that both *a* and *b* could have such an effect and yet act independently.

The striking factorial additivity of responses to *p*-nitrobenzoic acid, table 6 and figure 4, indicates that such cooperation can either raise or lower affinity. Both *a* and *b*, when alone, lead to moderate resistance to this drug. The highly sensitive *d* is brought back toward resistance by each of the factors *a* and *b*, or alternatively, one may say that a tendency toward greater reactivity can be superimposed upon relative resistance. This language might suggest that a merely passive, permissive acceptance of the 4-nitro group by *d* enzyme could not be manifested in the *ad* enzyme when accompanied by a positive restriction imposed by *a*. But, more precisely, these affinities express statistical probabilities of reaction and none is in reality more positive than any other.

When general factorial additivity is an established expectation, it becomes important to look for significant exceptions, where two markers are more—or less—effective when together than would be predicted. These are the cases which may reveal some overlapping of function in the enzyme properties affected by the two markers. None is found for SA or the sulfones in general, nor for the somewhat incomplete series of results in table 6.

There may be indications, however, of exceptional additivity for the substances presented in table 5. All doubly marked strains have 'excessive' resistance to *p-p'*-diaminobenzil (in relative resistance, *ad* is 55 instead of 14; *ab* is 24 instead of 2.6, etc.); the triplet *adb* shows a reasonable added resistance even above these levels. The aminoacetophenone shows indications of the same effects; for *p*-nitraniline the doubles are probably significantly less than 'additive'. The pair, *ab*, is the one which is most notably too high or too low, respectively. Without going too far into speculation, one might suppose that there is a significant progression in the following series of electronic shifts away from the PAB-like structure:

carboxyl (PAB) → nitro → sulfone → carbonyl (*groups*)

A → B → D → E (*field effects*)

nonadditive → weakly additive → additive → overadditive

Since, as discussed previously, there is no sign of any material difference in the affinities of the multiple strains

for PAB, they seem not to be 'additive' at all for their affinities for this important metabolite. The implication clearly would be that alterations of the structure which make the analog less and less like PAB in electron distribution will rather quickly render the substance incapable of reacting with PAB-utilizing enzyme—and different mutations (*a* and *b* in particular) of the enzyme structure can substantially reduce the probability of reaction. (The term 'reaction' in all of these discussions refers to a reversible binding at a specific site, but not the completion of an enzymatically catalyzed conversion.)

Nuclear-substituted analogs of PAB. Full application of the method of analysis by substrate affinities (29) makes use of the PAB derivatives bearing substituents on the aromatic ring. This phase of our work is by no means complete, but includes the study of some 23 compounds of this type. Some portion of the results which may be of interest will be presented now, not in tabular form, but in the discussion which follows.

Most remarkable is the special relation between strain *b* and *p*-aminosalicylate (PAS). This substance shows marked inhibition of growth, and folic acid synthesis, is *b* cells at 3 μg/ml concentration (and *b*/wild ratio = 3). But, we found that a very slow residual growth occurs even in 100 times this concentration, and in such conditions a folic acid is produced which pneumococci can, but *Str. fecalis* cannot, utilize for growth. The impression that PAS is directly incorporated by the *b* strain into a modified folic acid is strengthened by a report that carbon-14-containing PAS is accumulated in the folic acid fraction of enterococci (59).

This behavior toward PAS is relatively or exclusively a property specific to strain *b*, of our unit strains considered here, and is found to be carried over into the multiples containing factor *b*. Thus, *d* enzyme, sensitive to PAS (ratio 0.8–1) but not noticeably converting it, is further modified by the *b* factor in strain *db* to be an effective converter of PAS, although it is not clear whether it is a more efficient one than the enzyme of *b* cells. The factor *a* lends considerable resistance (8-fold) toward PAS, without leading to any pronounced ability to utilize this analog lacking further assistance.

The possibility seemed reasonable that a charge-specific component determining the affinity of the *b* family of enzymes was selective at the carboxyl position, for reasons implied before, but was associated with a steric rearrangement in the enzyme surface which allowed substitution at ring position 2 (occupied by hydroxyl in PAS see fig. 2). We have supporting observations which confirm the relationship, if not the hypothesis. When a 2-hydroxyl substitution is present in SA, the derivative is of low activity, but there are some signs of relatively greater activity toward the *b* enzymes. The same additional substitution upon 4-hydroxybenzoic acid reduces the activity upon *d*, but the *db* is almost as sensitive as the *d*. Still more convincing is the marked sensitivity of the *b*-modified enzymes to *p*-nitrosalicylate. The significant resistance which the *b* enjoys over wild type for both PAS and *p*-nitrobenzoate (ratios about 3) is reduced to a factor of 2 for *p*-nitrosalicylate, which moreover cannot be utilized. And it is noticeable that *b* conveys no additional resistance at all when combined with *a*, *d* or *ad*, quite in contrast to a fourfold effect of *a* in its multiples.

Since the 2-hydroxyl group of these compounds can form a hydrogen bond with the neighboring carboxylate, the comparison of 2-OH and 2-chloro and 2-methoxy substitution is being pursued. At this point it may suffice to summarize in full the findings for 2-chloro-4-nitrobenzoate. The chlorine atom has resulted in a reduced reactivity toward wild type of about 1/75th, and now the resistance ratios to wild type are: *a*, 1; *d*, 0.03; *b*, 0.65; *ad*, 0.13; *db*, 0.03; *ab*, 0.8; *adb*, 0.03. Besides the relative sensitivity of *b* (it had highest resistance to unsubstituted nitrobenzoate), one also sees that *b* cannot enhance the resistance of *a*, *ad* or the sensitive *d*. Thus, the wild type and *d* have suffered a fivefold larger reduction in reactivity than has *b*, with the introduction of the 2-chloro substituent into nitrobenzoate (*a* is somewhat intermediate). The indication is strong that *b* enzymes can accept certain groups at the 2-position of the ring, perhaps principally those with electron-holding atoms directly on the ring, or possibly any group of medium size.

There are some indications that *b* enzyme is inhibited by certain 3-substituted compounds as well, but the series of tests have not yet been completed or repeated. Similarly, the *a* strain is often slightly reactive toward 2- and 3-substituted compounds, although less so than *b*. One very challenging result, that has not been examined thoroughly for lack of material, indicates an interesting possibility of additivity between *a* and *b* to give heightened sensitivity toward a 2,3-disubstituted PAB, viz. 4-amino-1-naphthoic acid.[8] Since this response was shown only by two strains which contain *ab* (*ab* and *adb*), and not by *a* and *b* themselves, it is highly suggestive that we may soon be able to discern the nature of *a* and *b* and their cooperative effects upon the enzyme structure.

The remaining example we shall discuss will be that of methyl substitution upon the ring. The effect of a 2-methyl group upon PAB renders it so inert (as either inhibitor or metabolite) that one is not surprised to find the same substitution upon sulfanilic acid producing also an inactive analog. It may be supposed that the steric effect of the adjacent methyl group is sufficient to destroy whatever resonance and coplanarity the carboxyl or sulfonyl group enjoys with the ring. In this connection, the hydrogen bonding of the 2-hydroxyl may be envisioned as greatly supporting the coplanar nature of the benzoate ion and contributing to the activity of PAS.

On the 3-position a methyl group is not quite so

[8] We are particularly indebted to Dr. Robert G. Shepherd of the Research Division, American Cyanamide Co., for his generous contribution of a personally prepared sample of this rare compound.

destructive of activity. We have found 4-amino-3-methylbenzoate a substitute for PAB, weak but apparently competitive (after recrystallization) against SA. The *b* strain is especially responsive to this analog. Finally, 3-methylsulfanilic acid and 3,5-dimethylsulfanilamide are weak inhibitors but show a modified form of the familiar 'sulfone pattern' of activities against the various strains. We conclude that the 3-methyl substitution has either little effect, or not a highly important one, upon the adjacent amino group. The *b* enzyme is somewhat more likely to combine with analogs bearing such substitution than are the others.

Other aspects of substrate analysis. The last examples serve to bring up some final points concerning this method which we have called the substrate analysis of genetic modifications (29). The use of analogs which can substitute for PAB and be converted to products useful for the cell can reveal another set of structural requirements demanded by the chemical process of actual enzyme-substrate conversion. Also, the substances found to be inactive have eventually to furnish their negative evidence about the reactive sites. The control of the enzyme-substrate reaction by changing of pH has given suggestive results about enzyme properties, but these are considered tentative until a normal cell-free enzyme system is available. And even when it is, it will take a long time indeed to make rigorous comparisons of the many compounds that have already been examined on the several strains in the living condition. At least three other markers related to the Fa-d-b system are available and have been studied in part along the lines of the present investigation.

It might be useful to propose that in drug therapy, combined use of the two 'opposite' kinds of PAB analogs —sulfonamides and nitro- or hydroxy-benzoic acids, for example—might sharply restrict the development of resistant strains, if other pathogenic organisms have the same potentiality for mutational modification of their enzymatic system as do the pneumococci.

COMMENTS AND CONCLUSIONS

The genic fine structure genetically analyzed in the pneumococcal DNA seems to control an equally specific pattern of fine structure in the protein of a PAB utilizing enzyme (fig. 5). The relatively mobile—in transformation—DNA components of the genetic site can be made to move in or out, profoundly affecting what does or does not happen to the structure of the PAB-utilizing protein. There will of course, remain some margin of doubt about the identification of this protein until some sort of purification can be achieved, but it is not expected that this will lead to much greater understanding until and unless material can be prepared suitable for refined kinetic work and peptide analysis. It would be a challenging prospect to learn about the linear sequences of amino acids in a series of proteins which have such interesting and characteristic properties in their native folded forms.

A method of anlysis of the enzymatically active center has been devised, based upon systematic changes in structure of inhibitory analogs of the substrate, examined always against pairs of closely related strains, so that a basis for comparison is always present. Aspects of the protein fine structure can be described in fairly detailed and intimate terms for an enzyme not yet isolated, and not yet identified in the usual overall sense.

The subgenic factor *d* appears to modify the PAB-converting enzyme so that it becomes less reactive with inhibitory analogs containing bulky substituents occupying position 1 (carboxyl analogs) of the PAB structure. This holds for all sulfones and other groups of equal bulk; additional large substituents distal to this group reduce slightly further the reactivity. This phenotype is found in all four strains bearing *d* in the genotype. On the other hand, this phenotype becomes less exacting at the same time toward several analogs with different electronic distribution about position 4 (amino analogs). Markers *a* and *b* by contrast appear especially to modify charge distribution in the same enzyme so that it responds best to other particular electronic distributions closer to that of PAB. These field effects in the *a* and *b* enzyme and the less specific one of *d*, are different from each other and powerfully additive for substances electronically very different from PAB, less than additive for substances more nearly resembling PAB. Marker *b* simultaneously modifies the enzyme allowing it to accept small electron-rich groups at position 2 of the PAB ring, and somewhat less readily groups at position 3. The *a* phenotype shows these latter properties to a lesser degree, but can be potentiated considerably when combined with the phenotype determined by *b* marker.

It is remarkable that, although they interact, the unit genetic factors modifying this protein generally retain their quantitative characteristics virtually intact. Thus, the genetically independent units, *a*, *d* and *b* can cooperate to modify a single protein, which yet reveals the more or less independent quantitative contribution each subgenic determinant has made. Clearly we are here dealing with the very fundamental principles of gene expression. We seem to have demonstrated that parts of a gene have a point-for-point relationship to as many parts of a specific protein. This is surely one of the first times that specific and identifiable spatially distributed effects have been shown to result from several sites within a single genic region of a DNA molecule.

It is a pleasure to thank Dr. H. P. Broquist of the Lederle Laboratories and Dr. Robert Shepherd, American Cyanamide for the donation of samples of diaminobenzil, hydroxy-sulfanilamide, sulfanilylthiazole and several other valuable substances not mentioned above. Thanks also are due to Dr. R. U. Schock of Abbott Laboratories for 2-methye-4 aminobenzoic acid and several other derivatives. (The other chemicals were principally purchased from Eastman Organic Chemicals, Aldrich Chemical Company, or K and K Laboratories.)

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Genetic fine structure and enzyme formation[1]

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DURING THE PAST TEN YEARS clear proof that enzyme formation is genetically controlled has been obtained. This proof has come largely from the study of microorganisms and stems from the efforts of many investigators (1, 2). Extensive evidence has also been obtained indicating that the structural characteristics of the formed enzyme are genically determined (3, 4). Recent work additionally points to the conclusion that quantitative aspects of enzyme formation is an action of genetic material and is distinct from the action controlling the qualitative characteristics of the formed enzyme (5). It then appears that both the qualitative and the quantitative characteristics of enzyme formation are gene determined. The knowledge that enzyme formation is under the control of the genetic elements of the cell and that there exists an apparent one-to-one relation of gene to enzyme, viewed with the Watson-Crick model of DNA and the observed amino acid difference of genetically distinct hemoglobins (6), has led to the widespread belief that the base sequence of the DNA of the gene serves as a code, specifying the amino acid sequence of the formed enzyme. Alteration of the base sequence, in turn, is thought to alter the amino acid sequence and so results in the formation of an altered enzyme. This theory is of great interest at the present time and experimental evidence is in general agreement with such a postulation. It is known that mutation leads to formation of altered enzymes, though it has not been shown that the alteration results from an amino acid sequence alteration. While there is much that is in agreement there are odd bits and pieces of information which suggest that the theory may well be modified with time. It is obvious that a knowledge of the base sequence of a gene and the amino acid sequence of its enzyme would be of great interest. Progress in this field is being made. An understanding of gene and enzyme relationships, however, requires knowledge of sequences, and of genetic fine structure. Genetic fine structural analysis can serve as an excellent tool for directly relating gene structure to the enzyme, when a number of criteria characterizing diverse functions of the latter are available. It is the study of one gene and its product enzyme which will be reviewed here.

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The genetic control of tryptophan synthetase formation of *Neurospora crassa* will be reviewed. These studies have been in progress for a number of years and our present knowledge of this system stems from the investigation of a number of different investigators. We might first consider the enzyme. The enzyme, tryptophan synthetase, is required for the catalysis of the terminal step in the formation of tryptophan, i.e. the substitution reaction between indole-glycerol-phosphate (InGP) and serine. This reaction requires as a cofactor pyridoxal phosphate (7, 8). Tryptophan synthetase, however, can carry out two additional reactions (see fig. 1). It will catalyze the conversion of (InGP) to indole (reaction 3) and the conversion of indole to tryptophan in the presence of serine and pyridoxal phosphate (reaction 2) (7, 8). All three activities are associated with a single enzyme as fractionation of the enzyme does not alter the relative activity of the three reactions in question (4, 8, 9; and S. E. Ensign, unpublished observations). Thus, from purification studies there has been no indication of the relative enrichment or loss of one activity, as contrasted to the second or third. Mutational evidence gives additional strong support to the view that the three activities involve a single enzyme species.

Mutations affecting tryptophan synthetase are readily obtained. When tryptophan requiring mutants are isolated, one class is found to lack enzymatic activity for reaction 1. A large number of independently isolated mutants all of which are unable to form tryptophan and all of which lack detectable tryptophan synthetase activity, judged in terms of reaction 1, have been isolated. With such a group of mutants we can determine whether all of the mutations which have resulted in loss of this enzymatic activity are mutations in the same genetic region or are mutations at other regions as well. This can be determined by use of the inheritance test. Such experiments permit the clear conclusion that all of the mutations which have been isolated involve alterations of a restricted region of the genetic material of Neurospora (10). Thus, all of the mutations which have been isolated appear to be clustered in a single region of chromosome number 2 as indicated in figure 2. How complex is this region? Is it a region containing a single mutational site or is it a region containing many mutational sites, or, phrased in experimental terms, are all of the mutant strains which have been isolated identical or are functional differences observed between them? As mentioned earlier all of the mutant strains appear

similar in requiring tryptophan and in lacking enzymatic activity for reaction 1. If one scans these mutants using additional criteria, many differences are found. For instance, it is possible to determine whether a mutation, which has resulted in loss of enzymatic activity, has at the same time resulted in loss of ability to form a protein which is antigenically related to the parental enzyme. By preparing antibodies against the parental enzyme, mutant extracts can be scanned for the presence of material which will react with these antibodies. Such investigations reveal that mutant strains can be subdivided into two major groups on the basis of whether or not they lack a protein which will react with tryptophan synthetase antibodies. Strains lacking a cross reacting material are called CRM−, those having such a material are called CRM+. Detailed investigation of a CRM+ strain has indicated that the material found in at least this one mutant is serologically identical with the enzyme of the parental strain (12). Still other methods of comparison can be employed. Let us consider those strains which are CRM+. As was mentioned earlier all of the mutations which have been investigated to date result in loss of enzymatic activity judged in terms of reaction 1. However, what about reactions 2 and 3? Can mutations occur which result in the loss of reaction 1, but permit retention of reaction 2 or 3? The CRM's of various strains have been tested for their ability to catalyze reactions 2 or 3 and on this basis the CRM+ strains can be subdivided (see table 1). The majority of the strains which form a cross reacting material are found to lack enzymatic activity for reactions 1, 2, or 3. This is not true of all strains, however, since certain strains are found which while having lost the ability to catalyze reactions 1 and 2 retain enzymatic activity judged in terms of reaction 3 (8, 13). The CRM of these strains is active in catalyzing the conversion of InGP to indole. As might be expected, such mutant strains are characterized by their accumulation of indole during growth, in contrast to strains which accumulate the compound indole-glycerol. Thus, we have a second group of CRM+ mutants, a group which might be called indole accumulators. The indole accumulator strains in turn can be subdivided. Purified tryptophan synthetase does not show cofactor requirements for reaction 3, i.e. the InGP → indole reaction. Surprisingly this is not true of all the indole accumulators. If extracts of these mutant strains are purified, one finds that the CRM's of certain mutants are similar to the parental enzyme, i.e. are capable of catalyzing the conversion of InGP to indole in the absence of additional cofactors. However, a second group is found which is capable of catalyzing this reaction only if pyridoxal phosphate is present. Still a third group is found which is capable of carrying out this reaction only if pyridoxal phosphate and serine are both present in the reaction mixture (8). The role of pyridoxal phosphate and serine in this reaction is not known at present. It is possible to compare the affinity constant for serine in the mutant reaction with the affinity constant of serine for the indole serine reaction catalyzed by the parental enzyme. The affinity constants are found to be identical (8). It would appear that mutation has not affected serine affinity, and one can only suggest that serine may be required to permit InGP access to its active site. A novel mutant was discovered by Catcheside (15) which led to the realization that a third class of CRM+ mutants can be found. This group has lost enzymatic activity judged in terms of reactions 1 and 3, but has retained the ability to catalyze reaction 2 (14, 15). Such strains are able to grow on indole in place of tryptophan and are referred to as indole utilizing mutants.

One therefore finds that mutant strains, all of which have lost the ability to catalyze the conversion of indole-

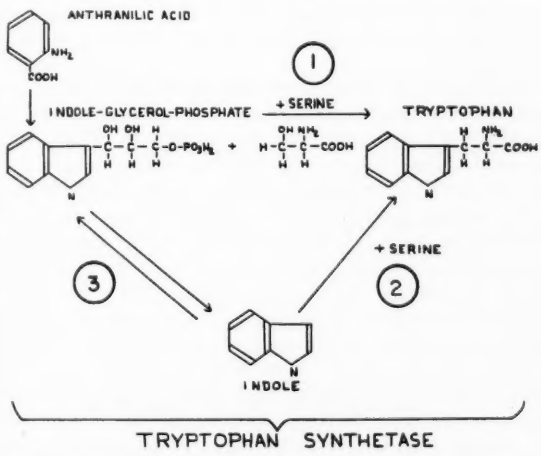

FIG. 1. Reactions catalyzed by tryptophan synthetase.

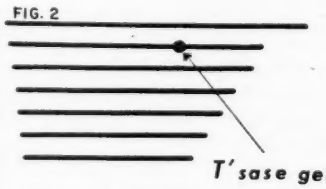

FIG. 2. The chromosomes of *Neurospora*.

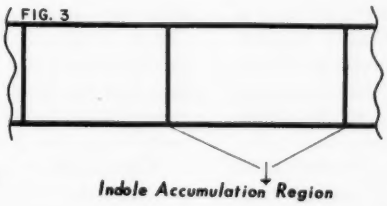

FIG. 3. Tryptophan synthetase gene showing regions in which mutations result in indole accumulation.

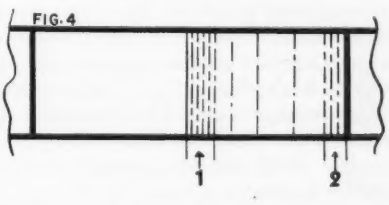

FIG. 4. Genetic fine structure of the indole accumulation region; *1* requires B_6PO_4; *2* requires B_6PO_4 + serine.

TABLE 1.

Functional Differences Found in Various Tryptophan Synthetaseless Mutants

I. CRM−
II. CRM+
a) Indoleless—reaction 1^-, 2^-, 3^-
b) Indole accumulator—reaction 1^-, 2^-, 3^+

	B_6PO_4	Serine
1	−	−
2	+	−
3	+	+

c) Indole utilization—reaction 1^-, 2^+, 3^-

glycerol phosphate to tryptophan, show many differences. Mutation may result in loss of ability to form a protein which is antigenically related to the parental strain. Mutation, however, may also result in the retention of ability to form a protein which is antigenically related to the parental enzyme, but a protein which is unable to catalyze any of the 3 tryptophan synthetase reactions. Mutation can also result in the loss of ability to catalyze reactions 1 and 2 but permit survival of reaction 3, or mutation may result in the loss of the ability to catalyze reactions 1 and 3, but permit the survival of reaction 2. Still other differences are found if mutants are compared in terms of temperature sensitivity or suppressibility (10, 16, 17). In any event, such investigations clearly point to the fact that the genetic region controlling the formation of tryptophan synthetase must be mutationally complex. It must contain many mutational sites, alteration of any one of which will result in a different functional alteration of the formed enzyme. We can enlarge the region of the chromosome controlling the formation of tryptophan synthetase and in the absence of other knowledge simply subdivide it indicating it as a region consisting of a large number of mutational sites. Obviously one would like to know something about the distribution of these sites within the genetic region as a whole. For instance, are the mutational sites arranged in linear array within the region, and do we find a clustering of mutational sites in terms of function, or do we find mutational sites which result in similar functional loss scattered at random throughout the genetic area as a whole? This problem can also be tackled by means of the inheritance test. Two tryptophan synthetase mutants of independent origin (alleles), if crossed with each other may form occasional tryptophan independent, or prototrophic, progeny. One can, therefore, prepare a recombination map in which these strains are mapped in terms of the relative frequency of prototroph formation in intra-allelic crosses. Inasmuch as the frequency of tryptophan independent progeny is low, a genetic analysis of this sort is based solely on the relative frequency of all recombinational events in diverse intra-allelic crosses.

We can now determine whether mutations which yield indole accumulators, i.e. mutations which have permitted the survival of reaction 3, appear to be distributed at random throughout the entire functional genetic region or whether they appear to be clustered. As contemporary theory suggests that mutation results in alteration of the primary structure and since folding characteristics are thought to be determined by the primary structure of the enzyme itself, one might a priori predict that mutations which result in similar functional alterations in a given end product should be distributed throughout the genetic area as a whole. This is not found to be the case (Y. Suyama and D. M. Bonner, manuscript in preparation). Genetic fine structure indicates that mutations which permit survival of reaction 3 are grouped in roughly one half of the genetic area as a whole, and thus do not appear to be at random (fig. 3). A more interesting relationship is found when we study recombination frequency within this group. Mutations which have resulted in a requirement for both pyridoxal phosphate, and serine appear to be clustered at one end of the tryptophan synthetase locus (Y. Suyama and D. M. Bonner, manuscript in preparation). Mutations which have resulted in a requirement for only pyridoxal phosphate also appear to be clustered at the other side of the indole accumulation region (fig. 4). Thus we find a region in which mutations result in retention of ability to catalyze reaction 3 but within this area mutations in one region impose a cofactor requirement for pyridoxal phosphate and serine, while in the second region they impose a requirement for pyridoxal phosphate alone. Scattered between these two major groups are those mutations which show no additional cofactor requirement.

A similar conclusion holds for mutations which have resulted in loss of reactions 1 and 3, but survival of reaction 2, i.e. indole utilizing mutations. The maximum frequency of recombination which has been observed in any intra-allelic cross involves an indole utilizing strain and an indole accumulator strain which requires both pyridoxal phosphate and serine. Mutations which have interfered with the ability of the enzyme to convert InGP to tryptophan but which have not interfered with the ability of the enzyme to catalyze the indole-serine reaction appear to be located at one end of the tryptophan synthetase locus (fig. 5). This observation was initially made with a single mutant strain, as this mutation has appeared only once. However, in reversion experiments as will be mentioned later, additional indole utilizing mutant strains have been obtained. These strains are clustered with the original mutant strain and like it show maximal rates of recombination in crosses with indole accumulator strains which require both pyridoxal phosphate and serine. Thus, as indicated in figure 5, we can delineate an additional region in our genetic map, a region which we can simply indicate as an indole utilization region.

What about mutations which are CRM+ but which have lost the ability to carry out reactions 1, 2, and 3? Such mutations are the majority class and are ones which might perhaps most logically be expected to be scattered at random. Surprisingly enough preliminary

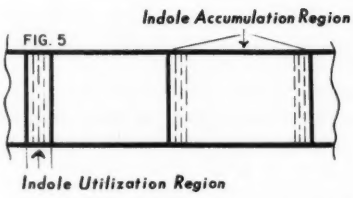

FIG. 5. Indole utilization region.

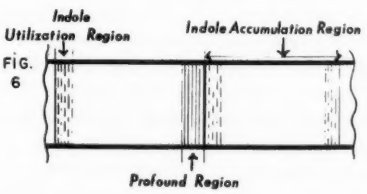

FIG. 6. The profound region.

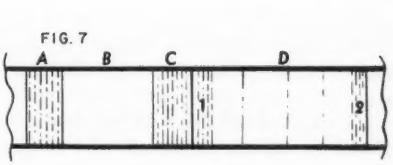

FIG. 7. Map of t'sase gene, April, 1960. *A.* Indole utilization-triose/InGP site. *B.* Quiet. *C.* Profound-indole site. *D.* Indole accumulation: *1.* $B6PO_4$ site. *2.* Serine site.

genetic analysis indicates that mutations of this group are clustered and are clustered in about the middle of the locus (fig. 6), a region called the profound region, as alteration has a profound effect. The last class that we have then to locate is the class which has not only lost all three enzymatic activities, but which at the same time has lost the ability to form a detectable CRM. Mutant strains of this type show aberrant recombination values characteristic of deletions. This aberrant recombination has been found characteristic of all of the CRM− strains. Not all of these strains result from deletion however, for were this the case, reversion to tryptophan independence should not occur, yet many of the CRM− strains which have been investigated are capable of reverse mutations to tryptophan independence. Recombination characteristics might then suggest that the reversible strains result from multisite mutation, i.e. in contrast to CRM+ strains, these strains might involve alteration at more than one mutational site. This suggestion is supported from reverse mutation studies of CRM− strains which appear to overlap the indole utilization region. Reverse mutation of such CRM− strains yield not only tryptophan independent strains, but also an equal number of strains which are still tryptophan requiring but which can now utilize indole in place of tryptophan. The frequency with which these two types of reverse mutations occur, however, make a multisite explanation unlikely. In fact at the present it is not possible to answer satisfactorily in all cases the nature of the genetic change which has resulted in the formation of the CRM− mutants. However, it is of interest to note that the recombinational characteristics of CRM− strains are distinct from those of the CRM+ and so suggest that alteration of a single mutational site may not yield a CRM− strain.

These data as a whole suggest numerous interesting possibilities. It is clear, that the genetic region controlling the formation of tryptophan synthetase can be divided into two major areas: a genetic area in which mutations appear to affect catalytic activity in reactions involving serine and pyridoxal phosphate, and a second region in which mutations appear to affect the enzyme affinity for InGP. Such a conclusion is not unreasonable in view of the finding by Crawford and Yanofsky (18) that the tryptophan synthetase of *Escherichia coli* will dissociate into two protein moieties, an A and a B protein. Neither the A nor the B protein has substantial activity for any of the three reactions by itself, but the A, B complex does show all three enzymatic activities. However, one of the two proteins again appears to be involved primarily in InGP affinity while the second protein appears to be primarily involved in those reactions involving serine and pyridoxal phosphate. At present we have no indication that the enzyme of *Neurospora* will dissociate into two independent protein fractions. In fact, all of the data at present is consistent with the view that this enzyme is a single moiety (9; S. E. Ensign, unpublished observations; and C. J. Wust, manuscript in preparation). This conclusion is of interest in view of the phenomenon of complementation in *Neurospora* (19–21). The tryptophan synthetase region of *Neurospora* consists of 5–6 complementation groups (15, 21). These complementation groups can be mapped, but inconsistencies between the complementation and recombination map are found. Complementation may not therefore measure discrete functional areas of the genetic region, but may rather measure interaction at the enzyme forming site itself.

The genetic map, however, is of interest for other reasons. This map suggests that mutational sites do not occur at random within the locus. Thus, mutations which can affect the ability of serine to react with indole or InGP are clustered. In view of the presumed dependence of the secondary and tertiary structures of an enzyme on its primary structure, such observations emphasize the need for further knowledge concerning protein structure. Of additional interest is the observation that while mutational sites are linearly arranged in the region they are not uniformly placed, gaps appear to exist (fig. 7). For instance there appears to be a genetically quiet region between the region in which mutations can result in loss of ability to carry out reactions 1 and 2, but permit survival of reaction 3, and the region in which mutation results in loss of the ability to carry out all three reactions. The relative size of this area, judged in terms of recombination, can only be guessed at, for the size of the region is a function of the number of genetic analyses which have been carried out. There is a clear possibility that what is indicated now as a mutationally quiet region may simply reflect the fact that insufficient mutants have been analyzed to date. However, the fact that there is this apparent gap does pose the possibility of there being regions within the gene in which mutations can occur and yet have no

substantial effect on the activity of the formed enzyme. This perhaps would be expected in view of the observed clustering of mutational sites. The very fact that mutations affecting specific catalytic activities are not found randomly distributed suggests that regions may well be found in which mutation and amino acid alteration could occur without affecting the catalytic characteristics of the formed enzyme. The region has been indicated as a mutationally quiet region rather than silent, for it is known that mutations which result in CRM− strains can occur within this region and thus mutational events can occur in this region and result in loss of enzymatic activity. Mutationally quiet regions pose a problem in terms of amino acid differences found between presumed mutant and parental enzymes. If mutationally quiet or silent regions do exist, the possibility of having substantial amino acid differences between diverse enzymes of identical enzymatic activity clearly exists. This could pose a thorny problem when comparing the amino acid composition of two catalytically similar enzymes of diverse genetic origins.

SUMMARY

Combined genetic and enzymatic study of tryptophan synthetase formation in *Neurospora crassa* indicate the following: The enzyme is capable of catalyzing the three reactions, *1*) a substitution reaction involving InGP and serine in the presence of pyridoxal phosphate, *2*) a reaction involving indole and serine in the presence of pyridoxal phosphate, and *3*) the conversion of InGP to indole. Mutation in the genetic region controlling the formation of this enzyme results in loss of catalytic activity for reaction 1. Such mutations, however, may result in the formation of an altered enzyme which may be active in reaction 2 or 3. Study of these mutants suggests that the genetic area concerned with tryptophan synthetase formation may be subdivided into two major regions. One of these two regions is concerned with the catalytic activities involving serine and pyridoxal phosphate and the second region is concerned with the catalytic activities involving InGP and indole. The former region may be subdivided into two major areas judged in terms of reaction 3, one in which mutations result in a co-factor requirement for pyridoxal phosphate and serine, and a second area in which mutations result in a cofactor requirement for pyridoxal phosphate. The second major region may be subdivided into two areas, one area in which mutations result in loss of enzymatic activity in all three reactions, and a second area in which mutations result in retention of catalytic activity in reaction 2.

These studies suggest that mutational sites are not randomly distributed in the genetic area which controls tryptophan synthetase formation. Rather mutations which are functionally similar appear to be clustered. Additionally, mutationally quiet regions in the locus appear to exist. These observations permit the construction of a genetic map of the tryptophan synthetase locus. Such a map poses problems of interest in the field of molecular coding and points to avenues of fruitful exploration for the future.

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SYMPOSIUM ON TOPICS OF CURRENT INTEREST IN BIOCHEMISTRY

Synthesis of nucleotides, nucleotide coenzymes and polynucleotides

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IN THE FOLLOWING, an attempt will be made to present briefly the principles of organic synthetic work in the general nucleotide field. More specificially, the topics to be covered are, firstly, methods for the synthesis of mononucleotides, then the problem of the synthesis of nucleotide coenzymes and related pyrophosphates, and finally, in a little more detail, the current work on the synthesis of polynucleotides.

SYNTHESIS OF MONONUCLEOTIDES

In the mononucleotide synthesis, we assume that the nucleosides themselves are available in a pure state and that therefore the chief problems in the synthesis of the nucleotides are those of *a*) chemical phosphorylation at a specific site in the molecule and *b*) since the phosphorylating agents, in general, lack specificity, a prerequisite to phosphorylation is the preparation of suitably protected nucleosides. Methods used commonly for preparing protected nucleosides will be illustrated with respect to the specific examples given below.

Phosphorylating agents. Chemical phosphorylation in the nucleotide field, which involves usually the transfer of an activated phosphoryl group to a hydroxyl group, has received the attention of many groups of workers during the last 40 or so years and a list of selected phosphorylating agents introduced at different times is given in table 1.

Phosphorus oxychloride used by Levene (27) and Gulland (14) and their respective co-workers usually gives very low yields and its major drawback lies in its polyfunctional nature. The monofunctional diphenyl phosphorochloridate introduced by Brigl and Müller (5) (see also Zeile, 51) is a very powerful reagent for phosphorylating hydroxyl groups but is not very useful in the nucleotide field because of the difficulty in removing the phenyl groups. Platinum catalyzed hydrogenolysis causes concomitant reduction of the purine or pyrimidine rings. Alkaline hydrolysis provides an alternative, but rather severe conditions are required. Dibenzylphosphorochloridate (52), although rather unstable and less powerful than diphenylphosphorochloridate, has proved relatively useful in the nucleotide field in the hands of Todd and his co-workers (1, 3). The reagent, tetra-*p*-nitrophenylpyrophosphate (32), the simple anhydride of di-*p*-nitrophenylphosphoric acid, is much more powerful and offers specific advantages in certain cases. The crude but simple method involving polyphosphoric acid (16) is also worthy of consideration in the phosphorylation of pyrimidine nucleosides and especially for the preparation of P^{32}-labelled nucleotides. Another powerful and general reagent which has recently been developed by Tener (13, 47) is provided by a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide. The procedure represents an ingenious application of the general method of phosphodiester bond formation discussed below in polynucleotide synthesis.

Nucleotide syntheses. Having introduced the different phosphorylating agents, selected examples will now be given of their use in the synthesis of mononucleotides using suitably protected nucleosides.

For the synthesis of ribonucleoside-5′ phosphates, the 2′- and 3′-hydroxyl groups of ribonucleosides are usually protected by the preparation of isopropylidene (I) or benzylidene derivatives. Adenosine-5′, uridine-5′, and cytidine-5′ phosphates have been prepared (3, 15, 16, 30, 32, 47) by phosphorylation of such derivatives by dibenzylphosphorochloridate and by other phosphorylating agents. In contrast, isopro-

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TABLE 1. *Reagents for Phosphorylation of Nucleosides*

Name and Formula	References
Phosphorus oxychloride $POCl_3$	14, 27
Diphenyl phosphorochloridate $(C_6H_5O)_2—P(=O)—Cl$	5, 51
Dibenzyl phosphorochloridate $(C_6H_5CH_2O)_2—P(=O)—Cl$	1, 3, 52
Tetra-p-nitrophenyl pyrophosphate $(O_2NC_6H_4O)_2—P(=O)—O—P(=O)—(OC_6H_4NO_2)_2$	32
Polyphosphoric acid $H_3PO_4 + P_2O_5$	16
β-Cyanoethyl phosphate + Dicyclohexylcarbodiimide $HO—P(=O)(OH)—OCH_2CH_2CN$ + $C_6H_{11}N=C=NC_6H_{11}$	13

pylidene guanosine (I; R = guanine) resists phosphorylation by a number of phosphorylating agents and until very recently[2] the only efficient chemical synthesis of guanosine-5′ phosphate was as illustrated in chart 1. Tetra-*p*-nitrophenyl pyrophosphate (II) is prepared *in situ* by reaction of the crystalline di-*p*-nitrophenylphosphoric acid with di-*p*-tolylcarbodiimide (32). Reaction with isopropylideneguanosine (I) at room temperature gives III, the di-*p*-nitrophenyl phosphate ester, in quantitative yield (9), from which the protecting groups may be removed as shown to give guanosine-5′ phosphate (VI). To be noted is the selective removal of one of the *p*-nitrophenyl groups and preparation of the diesters of the type V. Such compounds are proving useful in studies of phosphodiesterases (18).

In the deoxyribonucleotide field, the customary organic procedures are illustrated with respect to the synthesis of thymidine-5′ phosphate (chart 2). The reaction of thymidine with triphenylmethyl(trityl) chloride gives in excellent yield 5′-*O*-tritylthymidine (VII). Acetylation of the 3′-hydroxyl group, followed by treatment with 80% acetic acid, gives 3′-*O*-acetyl thymidine (IX) which is phosphorylated with dibenzylphosphorochloridate at low temperature in pyridine to give the dibenzylphosphate ester (X), from which the protecting groups can be removed as shown to give thymidine-5′ phosphate (XI) in high overall yield (11, 31).

Chart 3 illustrates the synthesis of thymidine-3′ phosphate (XIII) from 5′-*O*-trityl thymidine (VII) using a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide (abbreviated to DCC hereafter) (47). The example is chosen because the 3′-hydroxyl groups in these nucleosides are rather unreactive functions and this method is particularly effective for phosphorylation at these sites (see also under polynucleotide synthesis). The initial product is β-cyanoethyl phosphate ester (XII) from which the trityl and β-cyanoethyl groups can be removed by successive acidic and alkaline treatments to give XIII.

An alternative satisfactory synthesis of XIII, using yet a different phosphorylating agent, mono-*p*-nitrophenylphosphorodichloridate ($O_2NC_6H_4O—P(=O)—Cl_2$) has also been recorded (50).

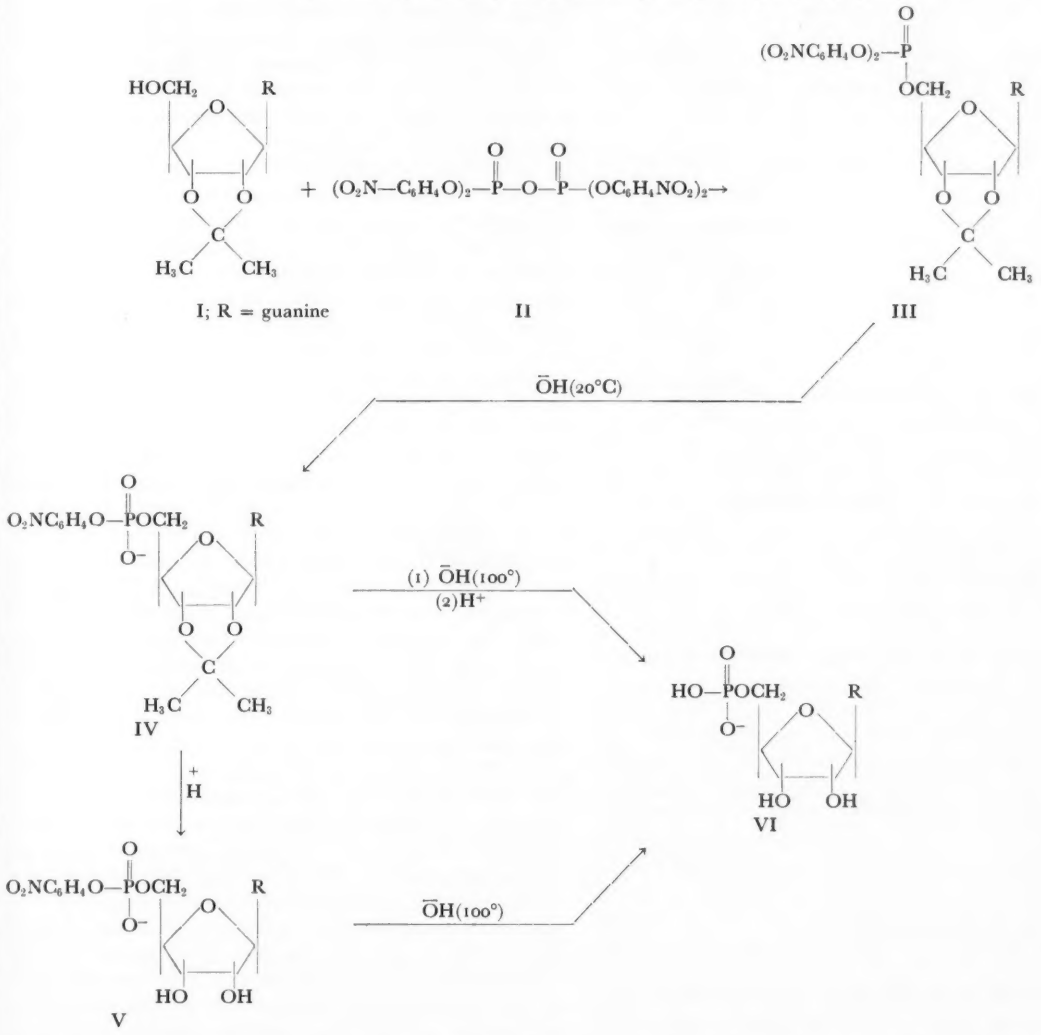
[2] Tener (47) has prepared guanosine-5′ phosphate in excellent yield by the phosphorylation of isopropylidene guanosine with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide.

SYNTHESIS OF NUCLEOTIDE COENZYMES AND RELATED COMPOUNDS

Nucleoside-5′ polyphosphates. The pioneering work in the field came from the laboratory of A. R. Todd (49) and the general approach, on which most of their early work was based, consists in the formation of a fully protected pyrophosphate followed by the removal of the unwanted groups. The approach is illustrated in chart 4 by the first synthesis of adenosine-5′ diphosphate (3). In recent years, the reaction of a mixture of two phosphate esters with DCC in aqueous pyridine has provided a simple one-step method for the synthesis of pyrophosphates (23). Chart 5 shows the postulated activated intermediate and the overall reactions leading to pyrophosphate synthesis. The method which was first developed for the synthesis of nucleoside-5′ di- and triphosphates (6, 15, 19) has been used widely (44) for a number of purposes, including the synthesis of aminoacylnucleotides. Although for the synthesis of nucleotide coenzymes more specific methods are now available, as described below, the general and improved procedure involving the condensation of nucleoside-5′ phosphates and orthophosphoric acid in the presence of tri-*n*-butylamine (41) still is the simplest and relatively efficient method for the synthesis of ribo- and deoxyribonucleoside-5′ triphosphates. The method has been used for the purpose in a number of laboratories (see e.g. 4, 37).

Specific synthesis of nucleotide coenzymes. The carbodiimide method discussed above lacks specificity in effecting condensation and satisfactory yields have been obtained in those applications where an excess of one of the two phosphate components can be used. Another serious limitation is encountered frequently in the synthesis of nucleotide coenzymes, where one of the components (e.g., D-glucose-α-1-phosphate necessary in the synthesis of uridine diphosphate glucose) bears an hydroxyl function suitably placed for an intramolecular cyclic phosphate formation (22). Consequently, the yields of desired unsymmetrical pyrophosphates have in general

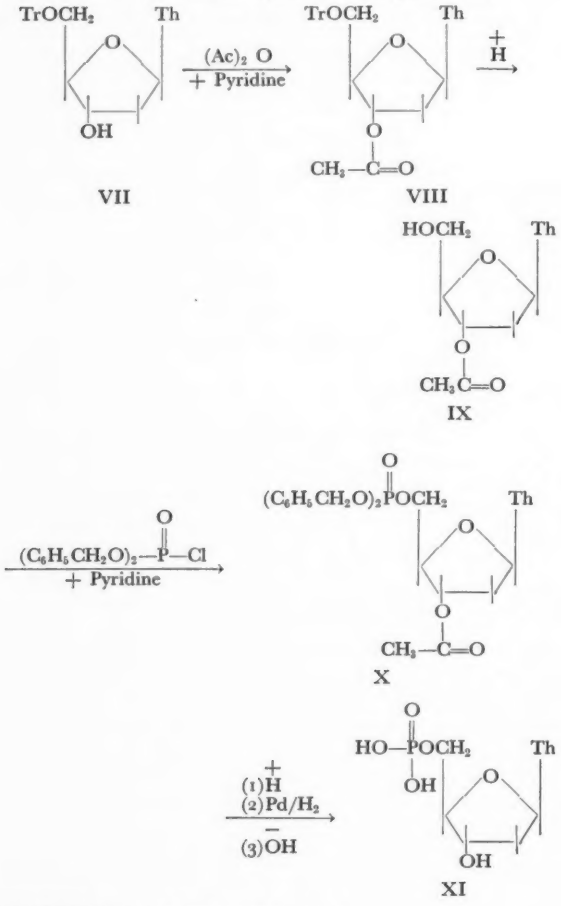
CHART 1. Synthesis of guanosine-5′ phosphate using tetra-*p*-nitrophenyl pyrophosphate.



been extremely low. Notable exceptions are the syntheses of a number of cytidine diphosphate compounds (26) and of diphosphopyridinenucleotide (17), all these compounds being free from the complications discussed above (cf. 44).

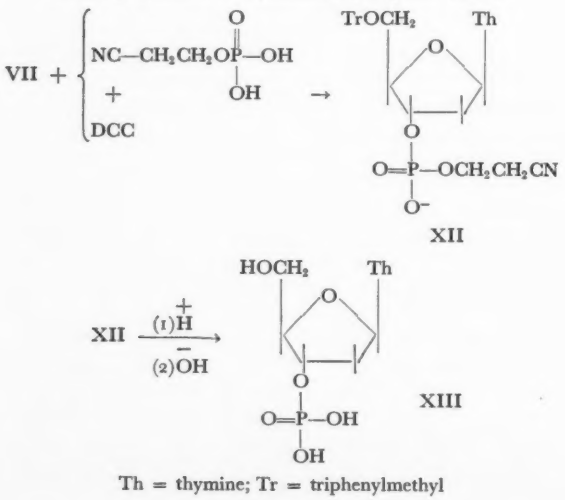
More recently, attention was focussed on specific methods for the formation of unsymmetrical pyrophosphates of the nucleotide coenzyme type (7). The work has culminated in carrying out the synthesis in two distinct steps. The first step involves the activation of the nucleoside-5′ phosphates to form nucleoside-5′ phosphoramidates (chart 6, step 1). The parent phosphoramidates obtained by condensation with ammonia (8) were used first (7, 33), but a more thorough investigation has shown the 5′-phosphoromorpholidates (XIV) to be the preferred intermediates (34) and a general procedure for their preparation in essentially quantitative yield has been described (35). For pyrophosphate synthesis, these substances, which are stable indefinitely, are then brought into reaction in dry pyridine at room temperature with the second phosphomonoester component, which is frequently the non-nucleotide part of the coenzyme (XV) (chart 6, step 2). The yields in all the numerous syntheses of nucleotide coenzymes (uridine-5′, cytidine-5′ and guanosine-5′ diphosphate compounds) that have been carried out by the standard procedure of chart 6 have been between 60 and 70% after isolation of pure products (33, 40). The important principle behind this generally satisfactory synthesis is that in step 2 (chart 6) the second phosphomonoester is utilized as an anion, i.e., the anionic oxygen attacks the electrophilic phosphorus of the phosphoromorpholidate; there is no activation of the phosphorus to oxygen bond in the phosphomonoester component and the intramolecular cyclic phosphate formation discussed above is avoided.

CHART 2. Synthesis of thymidine-5′ phosphate (XI).



VII, VIII; Tr = triphenylmethyl (trityl)
VII–XI; Th = thymine.

CHART 3. Synthesis of thymidine-3′ phosphate (XIII).



Th = thymine; Tr = triphenylmethyl

Of all the coenzymes of the nucleoside pyrophosphate group, coenzyme A represents easily the most complex of structures and a specific discussion of the work (chart 7) leading to its total synthesis (34, 36) is worthwhile because it is illustrative of much that we have learned about the chemistry of nucleotides as well as phosphate esters in general. Phosphorylation of adenosine with an excess of dibenzylphosphorochloridate gives as the main products the mixture of adenosine 2′ or 3′ and 5′-diphosphates (2, 10, 34, 36). The formation of these diphosphates and not 2′,3′,5′-triphosphate can be expected on the basis of more than one chemical argument (36). The direct reaction of the mixture of diphosphates (XVI) with morpholine and DCC does two jobs, and both surprisingly efficiently. The phosphomonoester group in the 2′ or the 3′-hydroxyl group bears an adjacent hydroxyl group and on being activated with carbodiimide gives the cyclic diester. When transported to this diester level, this function does not react further with the reagent under these conditions. The 5′-phosphomonoester group is quantitatively converted to the morpholidate. The net result of all this is the formation of the key intermediate (XVII) in 91–98% overall yield from adenosine diphosphates. When XVII is reacted with D-pantetheine-4′ phosphate (XVIII) under the standard conditions of pyrophosphate synthesis, only the phosphomonoester anion in XVIII attacks the electrophilic phosphorus of the phosphoromorpholidate. The cyclic diester anion in XVII is inert and this turned out to be a good way to have a potential phosphomonoester group in the 2′,3′-position. The main product is cyclic phosphate form (XIX) of coenzyme A, which is treated with mild acid to cleave the cyclic diester ring. The latter process occurs so as to give coenzyme A and the isomer (iso-coenzyme A) which bears the phosphomonoester group in the 2′-position. Chromatography of the total reaction mixture after work-up gives the mixture of coenzyme A and iso-coenzyme A in a combined yield of about 65% and the two isomers can be separated by rechromatography on another column. The synthetic coenzyme A thus obtained is fully active in the phosphotransacetylase system and identical in all respects with a purified sample from natural sources.

SYNTHESIS OF POLYNUCLEOTIDES

Method for synthesis of phosphodiester bonds. The approaches that have been investigated for the synthesis of phosphodiester bonds have been reviewed fully elsewhere (20). The method, which has proved so far the most satisfactory and on which essentially all of the existing work in the field has been based, is represented by *equation 1*. A phosphomonoester, such as a mononucleotide, is activated by DCC to form probably a metaphosphate type of product which serves as the phosphorylating agent for the hydroxyl function of the second component. No more need be said here about the mechanism of the reaction, which is obviously com-

CHART 4. Synthesis of adenosine-5′ diphosphate.

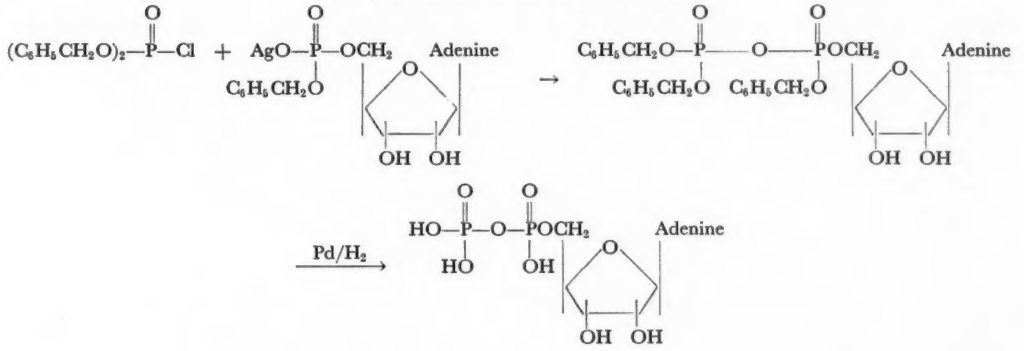
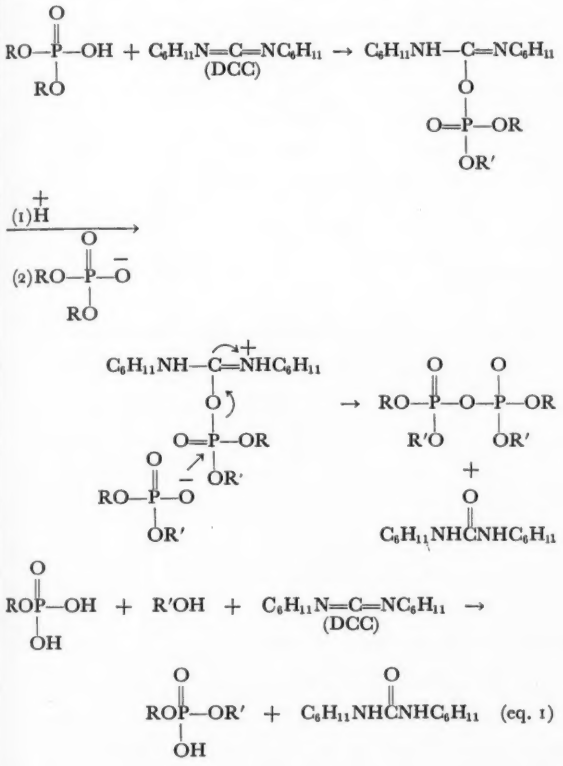


CHART 5. Pyrophosphate synthesis mediated by dicyclohexylcarbodiimide



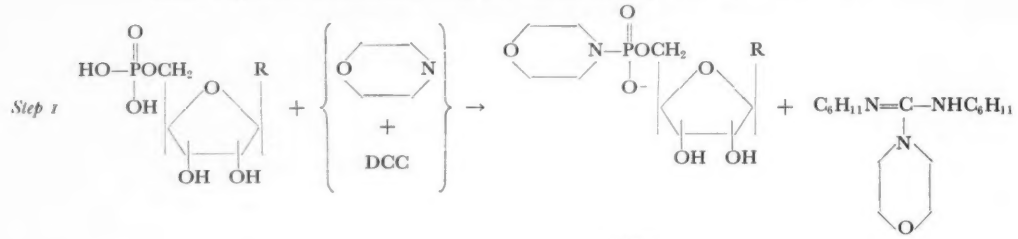
plex (11, 20, 44), except that the reaction must be carried out in an anhydrous medium and that satisfactory yields have consistently been obtained in all the cases studied.

Synthesis of internucleotide ($C_{3'}-C_{5'}$) *bonds between deoxyribonucleotides.* Analogous in many ways to the principles evolved in peptide synthesis, the formation of internucleotide bonds (chart 8) requires as the first step the protection of 'extra' reactive functions. Thus the 3′-hydroxyl group in thymidine-5′ phosphate is protected by conversion to the 3′-acetylated derivative (XX); in the second component, the 5′-hydroxyl group is protected by a triphenylmethyl group (see VII, above). Subsequent activation of the phosphomonoester group now gives specifically the $C_{3'}-C_{5'}$ linked compound (XXI) in about 70% yield (11). If an excess of one of the components over the second is used, then the yield of the diester is practically quantitative (unpublished work). The removal of the protecting groups to give XXII can be effected smoothly as shown in chart 8. By essentially the same procedure, but by replacing XX with protected deoxycytidine-5′ phosphate and deoxyadenosine-5′ phosphate, satisfactory yields of deoxycytidylyl-(5′→3′)-thymidine and deoxyadenylyl-(5′→3′) thymidine, respectively, were obtained (11). When attempts were made to vary the protected deoxyribonucleoside component in the above synthesis (e.g. replacement of VII by 5′-*O*-triphenylmethyldeoxycytidine) the preferred reaction was the phosphorylation of the N^6-amino group. Selective protection of the amino groups in the heterocyclic rings of nucleosides clearly became one of the prominent problems (see below). Satisfactory approaches have now been found (see also below). For synthesis of the type illustrated in chart 8, N^6-benzoyl-5-′-*O*-triphenylmethyldeoxycytidine (B. Lerch and H. G. Khorana, unpublished work) was prepared and condensed with XX. Thymidylyl-(5′→3′)-deoxycytidine was thus obtained after removal of the protecting groups.

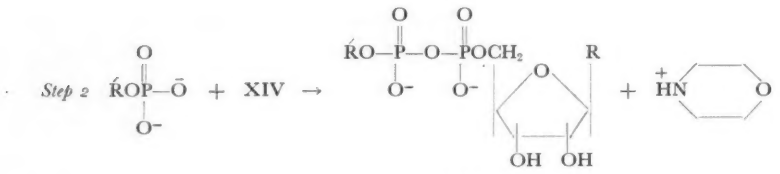
An alternative general approach for the inter-deoxyribonucleotide bond formation is to utilize appropriately protected deoxyribonucleoside-3′ phosphates. This has been done (G. Weimann and H. G. Khorana, unpublished work) in the synthesis of XXII by the scheme outlined in chart 9. (See also below under interribonucleotide bond synthesis for use of protected ribonucleoside-3′ phosphates.)

Stepwise synthesis of mixed deoxyribo-oligonucleotides. Stepwise synthesis of oligonucleotides containing different mononucleotide units requires selective exposure of an hydroxyl function at one end of the fully protected dinucleoside phosphates (e.g. XXI) to give substances like XXIII and condensation with another suitably protected mononucleotide. So far two trinucleotides

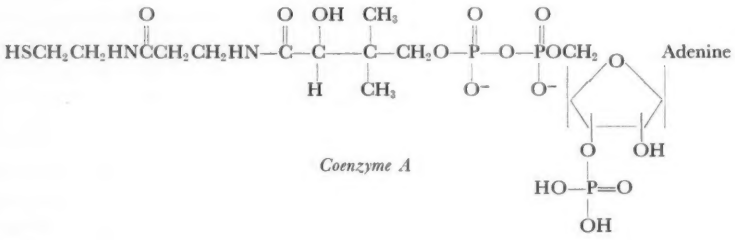
CHART 6. Synthesis of nucleotide coenzymes via nucleoside-5′ phosphoromorpholidates.



XIV

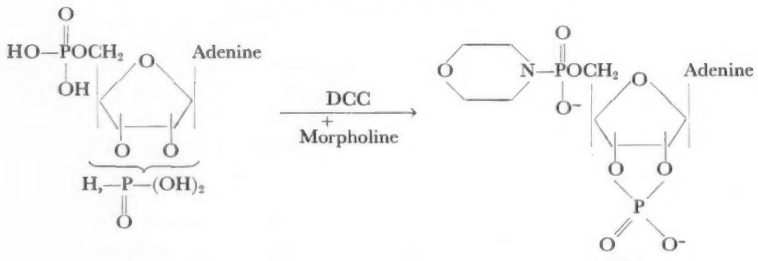


XV

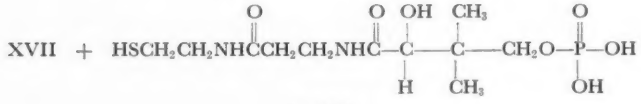


Coenzyme A

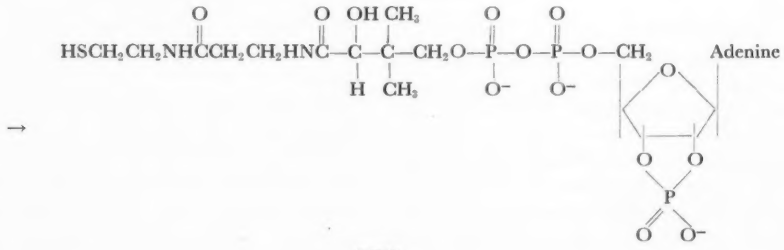
CHART 7. Synthesis of coenzyme A.



XVI XVII



XVIII



XIX

XIX $\xrightarrow[(2)\ HSCH_2CH_2OH]{(1)\ O\cdot INHCl}$ Iso-Coenzyme A + Coenzyme A.
(3) Chromatography

CHART 8. Synthesis of thymidylyl-(5′ → 3′)-thymidine.

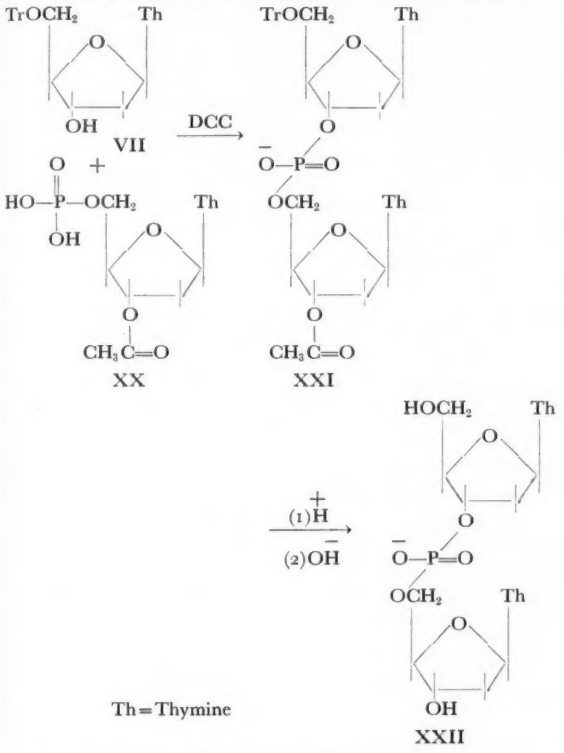
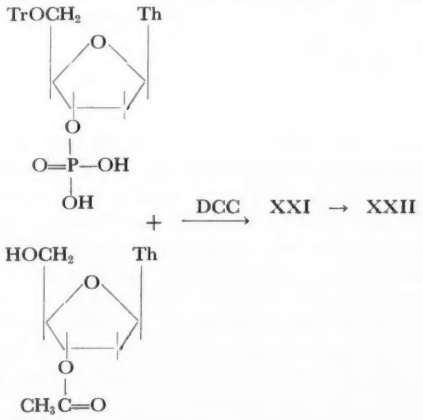


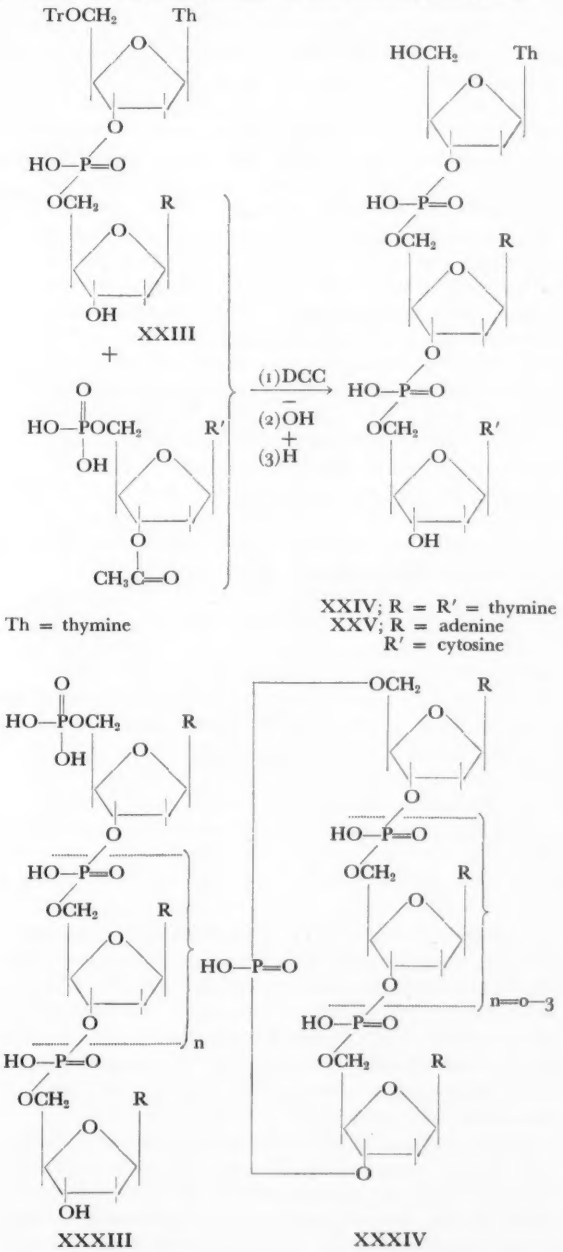
CHART 9. Alternative synthesis of thymidylyl-(5′→3′)-thymidine.



have been prepared this way (chart 10). These are thymidylyl - (5′→3′) - thymidylyl - (5′→3′) thymidine (XXIV) and deoxycytidylyl - (5′→3′) - deoxyadenylyl - (5′→3′)-thymidine (XXV) (12). Synthesis of higher and various mixed oligonucleotides is certainly feasible and is in progress. One serious problem has been recognized as the interference from the amino groups on the adenine and cytosine rings. However, as mentioned above (see also below), promising approaches to the problem now exist.

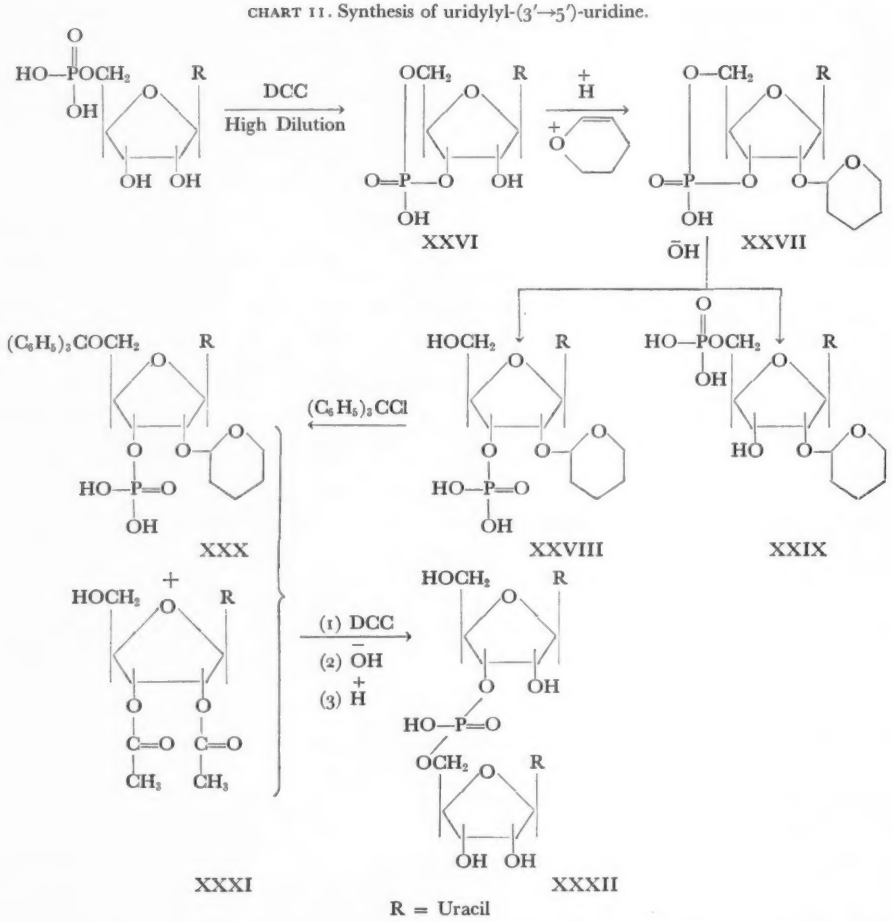
Introduction of phosphomonoester groups at termini. Linear

CHART 10. Stepwise synthesis of deoxyribo-oligonucleotides.



polynucleotides usually carry a phosphomonoester group at one or the other end of the chain and the methods described can be adapted to yield such 'true' oligonucleotides. Two approaches are available. The first one is that in which a protected phosphoryl group is used as a blocking group on a nucleoside at the outset of the synthesis (e.g. dibenzylphosphoryl group in place of triphenylmethyl group in VII). A number of dinucleotides bearing 5′-phosphomonoester groups have been

CHART 11. Synthesis of uridylyl-(3′→5′)-uridine.



synthesized in this way (11). In the second approach, the phosphomonoester end group is introduced after the formation of the internucleotide bonds (11). Thus, for example, XXIII (R = thymine) has been phosphorylated by a mixture of β-cyanoethyl phosphate and DCC. Subsequent removal of protecting groups gives dithymidine dinucleotide bearing 3′-phosphomonoester end group (47).

Specific synthesis of $C_{3'}$—$C_{5'}$ *inter-ribonucleotide linkage.* The specific synthesis of $C_{3'}$—$C_{5'}$ inter-ribonucleotide linkage is seriously complicated by the cis-(2′)-hydroxyl group in the ribonucleosides. The requirement of any general approach to the problem is to selectively block the 2′-hydroxyl function with a group which will not migrate to the 3′-hydroxyl function and which can be removed at the end without damage to the phosphodiester linkage synthesized. Such an approach is outlined in chart 11 and was recently used successfully (42) in the first synthesis of a $C_{3'}$—$C_{5'}$ linked di-ribonucleoside phosphate (XXXII). Uridine-5′ phosphate is converted in high yield to the 3′,5′-cycle phosphate (XXVI) on reaction in dilute solution with DCC (43). Reaction of the cyclic phosphate, as the free acid, with dihydropyran gives quantitatively XXVII, which hydrolyzes in alkali to give a roughly 4:1 mixture of XXVIII and XXIX. Although the mixture can probably be separated on an ion-exchange column, direct treatment with triphenylmethyl chloride in pyridine selectively converts XXVIII to XXX and the latter is readily separated from XXIX by partition chromatography. The condensation of XXX with 2′,3′-di-*O*-acetyluridine (XXXI) followed by removal of protecting groups gives XXXII exclusively.

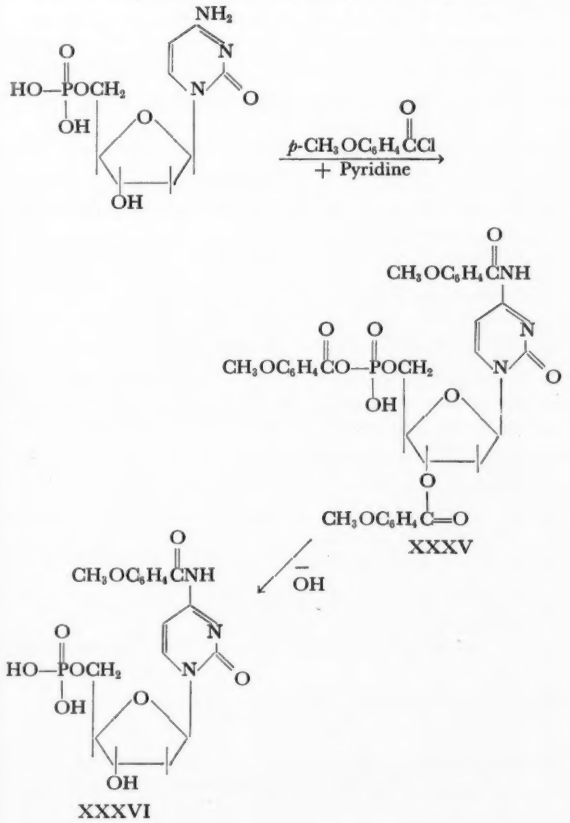
In further work with the above method uridylyl-(3′→5′)-adenosine has been synthesized by using an appropriately protected adenosine derivative in place of XXXI (45). The derivative bearing only the 5′-hydroxyl group free was prepared by benzoylating 5′-*O*-tri-(*p*-methoxyphenyl)-methyl adenosine with benzoyl chloride and subsequently removing the highly acid-labile tri-(*p*-methoxyphenyl)-methyl group under very mild conditions. The synthesis of higher ribooligonucleotides is in progress.

Polymerization of deoxyribo-mononucleotides. In the above

method of phosphodiester bond synthesis it was to be expected that treatment of an unprotected nucleotide (bearing both an hydroxyl group and the phosphomonoester group) with DCC under the standard conditions would cause polymerization. Thymidine-5′ phosphate was used in initial studies and polymeric products were indeed obtained. (Reactive anhydrides such as *p*-toluenesulfonyl chloride also bring about polymerization.) The separation and characterization of the products have been described in detail (48) and here it is sufficient to say that two series of homologous polynucleotides were obtained. The first series are the 'linear' polymers, containing the repeating naturally occurring phosphodiester linkage, a 5′phosphomonoester group at one end and a 3′-hydroxyl group at the other (general structure, XXXIII). The second series of compounds are the cyclic oligonucleotides (XXXIV) that arise by end-to-end intramolecular cyclization of the linear polymers. The extent of the cyclization process which competes with linear polymerization decreases with increase in chain length (48).

In more recent work (21, 25) on the polymerization of thymidylic acid, attention has been focussed on two aspects of the problem. In the first, aim has been to minimize the formation of cyclic compounds(XXXIV).

CHART 12. Synthesis of N^6-anisyldeoxycytidine-5′ phosphate.



This is accomplished to a great extent by incorporating some 25% of 3′-*O*-acetylthymidine-5′ phosphate in the unprotected mononucleotide. The protected nucleotide forms the terminating unit of the greater portion of the resulting polynucleotide chains and these are then unable to undergo the cyclization reaction. The acetyl group is subsequently removed by mild alkali. Using this technique and working in concentrated solution (25), the major products obtained are linear polynucleotides. Secondly, much effort continues to be devoted to the development of techniques for the rapid separation, isolation and characterization of the synthetic products. Recently (25), procedures have been described for the complete purification of members up to dodecanucleotide on a fairly preparative scale. A technical advance here has been the use of cellulose anion exchange columns in the carbonate form (46) and the use of the volatile triethylammonium bicarbonate (38) as the eluent.

The above-described principle of adding a protected mononucleotide before polymerization can be used in preparing another useful series of compounds, homopolymers terminated in a different nucleotide. Thus, by copolymerizing N,*O*-diacetyldeoxycytidine-5′ phosphate and thymidine-5′ phosphate, linear thymidine polynucleotides terminating in deoxycytidine residues have been prepared (25).

While the isomeric thymidine nucleotide, thymidine-3′ phosphate, could also be polymerized (50) by the above method, difficulties were encountered in extending the work to other deoxyribonucleotides. The problems were *1*) the reactivity of the amino groups in the heterocyclic rings (see also above) and *2*) the insolubility of the mononucleotides in anhydrous pyridine, which so far is the only satisfactory medium for polymerization. Both types of problem have now been solved by preparing N-acyldeoxyribonucleoside-5′ phosphates, the N-acyl groups used conferring adequate solubility on the nucleotides (24, and unpublished work of R. K. Ralph and H. G. Khorana). The procedure developed for the preparation of N-acyl derivatives is simple, efficient, and general, and may be illustrated with respect to N^6-anisyldeoxycytidine-5′ phosphate (XXXVI) (chart 12). Anisylation in pyridine at room temperature gives, presumably, the trianisyl derivative (XXXV) in quantitative yield. Carefully controlled alkaline treatment converts the latter to XXXVI, again in practically quantitative yield. The product is directly used for the polymerization reaction. The resulting small-sized deoxycytidine polynucleotides, after the removal of the protecting group, have been separated and characterized (24). Deoxyadenosine polynucleotides have also been synthesized by using N-benzoyl deoxyadenosine-5′ phosphate (R. K. Ralph and H. G. Khorana, unpublished work), the latter being prepared essentially as described above for XXXVI. Similar techniques are being applied to the polymerization of deoxyguanosine-5′ phosphate.

Ribopolynucleotides containing random C_2'–C_5' *and* C_3'–C_5' *internucleotide bonds.* The application of the above polymerization procedure to uridine-5′ phosphate gives

polymers which are obviously oligonucleotides containing random mixtures of $C_{2'}$—$C_{5'}$ and $C_{3'}$—$C_{5'}$ internucleotide bonds (44). Michelson (28, 29) has reported on the formation of ribopolynucleotides containing random internucleotide linkages by further activating ribonucleoside-2′,3′ cyclic phosphates with diphenylphosphorochloridate. Although no adequate characterization or isolation of pure individual polynucleotides has been reported so far, there is little doubt that small-sized randomly linked polynucleotides were obtained.

In the near future, the synthesis of ribopolynucleotides containing specifically the $C_{3'}$—$C_{5'}$ internucleotide bonds may be expected by polymerization of suitably protected derivatives of the types XXVIII and XXIX.

CONCLUDING REMARKS

From the above brief review, it is evident that a range of satisfactory methods of phosphorylation is available for the synthesis of mononucleotides; the situation as regards the specific synthesis of unsymmetrical pyrophosphates (nucleotide coenzymes) may also be pronounced satisfactory. Attack on the problems of the chemical synthesis of polynucleotides is of more recent origin but the work has already evolved concurrently along several distinct lines. In brief, there are the two obvious divisions into ribopolynucleotides and deoxyribopolynucleotides. Each of these fields is further divided into *a*) stepwise and specific synthesis of polynucleotide chains with predetermined sequence of mononucleotide units, and *b*) polymerization to form polynucleotides.

Synthesis in the nucleotide field has come to assume functions much wider than those assigned to synthesis in classical organic chemistry. For example, the pioneering work of Kennedy and co workers (26) on phospholipid biosynthesis using cytidine diphosphate compounds was carried out with samples prepared by chemical synthesis before the compounds were isolated from natural sources. The situation is even more pertinent in the polynucleotide field. Chemical structural studies, physico-chemical studies, elucidation of the mode of action of enzymes of nucleic acid metabolism (18, 39) and, in short, understanding of the biological functions of the nucleic acids in precise chemical terms will all be profoundly influenced by polynucleotide synthesis. In the distant future, the total chemical synthesis of macromolecules possessing biological function must also be considered. The problems are vastly more complex than anything previously undertaken by experimental organic chemistry Nevertheless, Biology urgently asks: Will Organic Chemistry extend its horizons and accept the challenge?

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Bioluminescence

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Light emission occurs from a number of chemical oxidation reactions. Bioluminescence is an example of chemiluminescence in which the organic, oxidative reaction is catalyzed by an enzyme. Workers concerned with the mechanism of light emission by organisms have looked to the nonenzymatic reactions of ordinary chemiluminescence for suggestions concerning the mechanism of the interconversion of chemical energy and light energy. Unfortunately, chemiluminescence reactions are very inefficient and consequently most light-emitting reactions cannot be studied quantitatively because of the relatively large proportion of products formed from dark reactions. It may be therefore that the highly efficient enzyme catalyzed reactions might be more amenable to analysis than most other chemiluminescent processes.

So far our analysis indicates that the requirements for chemiluminescence and bioluminescence are an ionizable substrate which could form a peroxide addition, molecular oxygen and a fluorescent intermediate or product molecule. In bioluminescence the enzyme binds the substrate in such a manner that a catalyzed oxygen addition could occur at the proper ionizable site. In addition, the enzyme protects the excited state from nonluminescent quenching interactions. Thus it appears that the enzyme-substrate complex is extremely important for intramolecular energy transfer which will determine the efficiency of light emission and in some cases the color.

In the present brief review the data available which which bear on the mechanism of a well known chemiluminescent reaction (luminol) and a bioluminescent reaction (firefly) will be summarized.

Luminol

The oxidation of luminol (5-amino-2, 3-dihydro-1, 4 phthalazinedione) is one of the most efficient chemiluminescence reactions and has attracted the attention of a number of investigators since the discovery of the light emitting process by Albrecht in 1928 (1). Recently White (6) and associates and Seliger (3) have reinvestigated this reaction in great detail and I would like to review some of their findings, since the proposed reactions clarify some of our ideas concerning mechanism.

Normally the luminol reaction has been studied in a system composed of water, hydrogen peroxide, strong KOH and some activating agent such as potassium ferricyanide or a transition metal. Hemin is excellent. A chelating agent is usually used to prevent the precipitation of the metal oxide or hydroxide.

White's scheme, presented in figure 1, for the luminol reaction in aqueous medium is probably the most reasonable as to the mechanism of light emission. As White points out there is no unambiguous experimental evidence supporting any of a number of mechanisms. The facts available indicate that *a*) oxygen is required, *b*) the anion of luminol is the reactant, *c*) free radicals are involved and *d*) nitrogen is a product of the reactions.

The study of the chemiluminescence in water systems is not too promising because of the dark destruction of the intermediates and degradation of the end products of the light reaction by the powerful oxidizing agents. Until recently the highest quantum yield measured for a water system has been found to be 0.003 light quantum emitted per luminol molecule oxidized. It is evident therefore that the luminol molecule is readily destroyed in dark reactions, and to distinguish these steps from

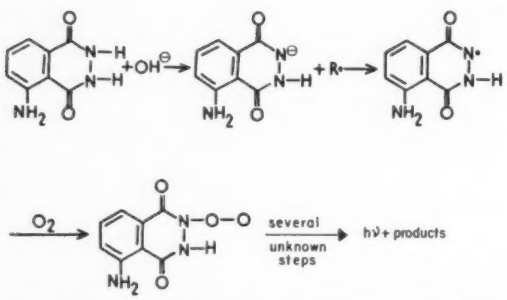


FIG. 1. Reaction scheme for the chemiluminescence of luminol in water.

the light-emitting processes is most difficult if not impossible.

Recent evidence by Seliger has indicated that pH is an extremely important factor in determining quantum yield. In addition, his results support the suggestion made by White that in aqueous solution the emitting molecule is most likely from the excited oxidized product molecule, possibly the ion of amino phthalic acid.

Figure 2 shows the emission spectrum of luminol chemiluminescence and fluorescence. Since the two curves give a peak at 430 mμ and are nearly identical, a number of workers and in particular Spruit and Spruit Van der Burg (5) have suggested that the emitting species is the neutral luminol molecule. This would imply an energy transfer from the excited oxidation product to a neutral luminol molecule. If this were true there should be a dependence of the chemiluminescence quantum yield on luminol concentration. Seliger does not find this to be the case. A further argument against luminol being the emitter is presented in figure 3. The fluorescence quantum yield of the luminol molecule is plotted as a function of pH. At precisely those pH values where chemiluminescence occurs the fluorescence quantum yield of the 430 mμ emission decreases practically to zero. Similar studies by Seliger using pure amino phthalic acid are presented in figure 4. The fluorescence quantum yield for this compound is highest in the pH range between 8 and 10 but decreases rapidly as the pH is raised toward 14. This correlates reasonably well with the chemiluminescence quantum yield measured at different pH's. Seliger has found that at pH 9.6 the quantum yield for luminol chemiluminescence is 0.01, pH 11-0.02, pH 13-0.006 and in 10 N NaOH this yield was 0.0004. In addition the chemiluminescence emission spectrum in 0.1 N NaOH corresponds very closely to the fluorescence emission of amino phthalic acid under the same conditions (fig. 5). These correlations between the quantum yields and emission spectra support the suggestion that the excited amino phthalic acid is a good candidate for the emitting molecule. The additional fact that the chemiluminescence quantum yield is independent of the luminol concentration seems adequate justification to eliminate the neutral molecule or the mono-anion as the emitting molecule. Intermolecular energy transfer in this reaction is therefore not a necessary or a likely possibility.

CHEMILUMINESCENCE IN ORGANIC SOLVENTS

When luminol is placed in an organic solvent such as dimethyl sulfoxide under alkaline conditions, light emission occurs without the addition of hydrogen peroxide. White has shown that the final products of the reaction are nitrogen and 3-aminophthalic acid. The chemiluminescence quantum yield for this reaction as measured by Seliger is about 0.1, using the tertiary butoxide ion to initiate the reaction.

With a strong base in the organic solvent, luminol is converted into the dianion which reacts with one molecule of oxygen to liberate nitrogen and to give the excited state of either the dianion of aminophthalic acid or its immediate precursor. The hydroperoxide ion shown in figure 6 is a possible intermediate.

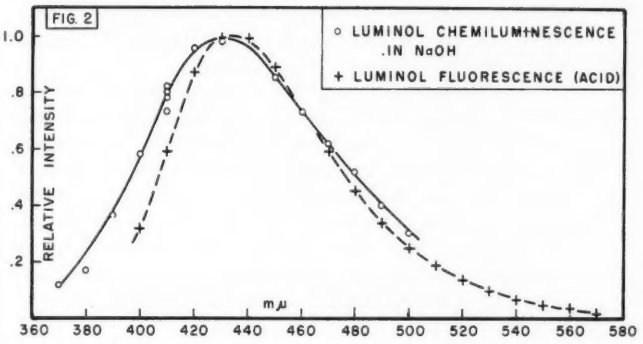
The luminol light emitting reaction in dimethyl sulfoxide has an emission peak at 480 mμ which corresponds to about 60 Kcal. per einstein. White has calculated that over 90 Kcal. are available in the proposed decomposition of luminol. Since most of the energy liberated occurs at the time of the formation of the nitrogen molecule it suggests that the excited amino phthalate ion or an immediate precursor is the emitter. White has suggested the pathways shown in figure 7. However, the fluorescence emission spectrum of the amino phthalate ion in dimethyl sulfoxide does not agree with the chemiluminescence emission. This is also true of the fluorescence in other organic solvents such as dimethyl formamide which will also support the luminol light-emitting reactions. Clarification of the actual emitter must await further work.

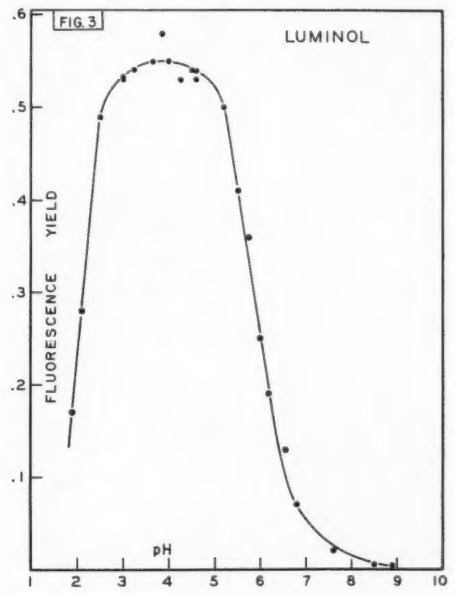
FIREFLY LUMINESCENCE

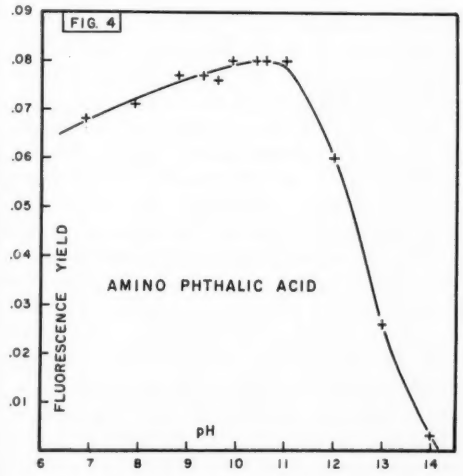
The factors required for light production in extracts from fireflies have now been prepared in a highly purified crystalline state. The crystallization of firefly luciferase and luciferin has allowed for the first time an extensive quantitative study of the interaction of these substances during the process of light emission (2).

The exact structure of the highly fluorescent luciferin molecule is not completely known. The empirical formula is $C_{13}H_{12}N_2S_2O_3$ and a great deal of evidence indicates that it is a benzothiazole derivative. It is well established that the luciferase catalyzed reaction between luciferin and adenosine triphosphate (fig. 8) leads to the formation of an intermediate enzyme luciferyl-adenylate complex (E-LH_2-AMP) and pyrophosphate (POP). Light is produced when in the presence of oxygen, E-LH_2-AMP is oxidized to the enzyme-oxyluciferyl-adenylic acid complex (E-L-AMP).

A large amount of data indicate quite clearly that the oxidized luciferyl-adenylic acid derivative is tightly complexed to the enzyme which effectively removes the







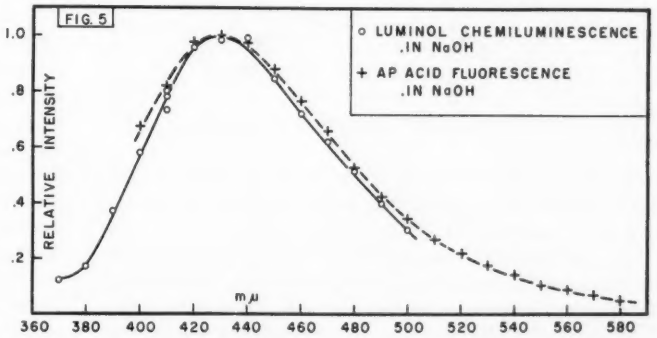


FIG. 2. Chemiluminescence and fluorescence spectrum of luminol.

FIG. 3. Fluorescence quantum yield of luminol as a function of pH.

FIG. 4. Fluorescence quantum yield of amino phthalic acid as a function of pH.

FIG. 5. Chemiluminescence of luminol and fluorescence of amino phthalic acid in 0.1 N NaOH.

latter from catalytic activity. Low concentrations of pyrophosphate react with the enzyme-oxyluciferyl-adenylic acid complex to give free enzyme, ATP and free oxyluciferin. The free enzyme in the presence of ATP and oxygen is now available to catalyze the oxidation of a second luciferin molecule.

Probably the best evidence for the proposed scheme in figure 8 is the demonstration that LH_2-AMP formed chemically by the condensation of LH_2 and AMP can act as an active substrate in the presence of the enzyme and without ATP or magnesium ion to produce light. Starting with LH_2-AMP and crystalline luciferase in solution the only other requirement for light emission is oxygen. Free oxyluciferin has no inhibitory effect on the rate of light emission. However, ATP gives a slight inhibition of the rate of light emission which becomes much more pronounced in the presence of magnesium ion.

The product of the enzymatic light reaction initiated with LH_2-AMP has been identified as the oxyluciferyl-adenylate derivative.

The reversal of the two activating reactions outlined in figure 8 by pyrophosphate has actually been shown by demonstrating a net synthesis of ATP as well as the incorporation of P^{32}-labeled pyrophosphate into ATP. That L-AMP is the inhibitor of luciferase is clearly shown when chemically synthesized L-AMP is added to a light-producing reaction. Because of the very tight binding of the oxyluciferyl-adenylate to the enzyme equi-molar amounts of L-AMP and enzyme give no light production in the presence of ATP and luciferin. It is of interest in this connection that the chemically synthesized

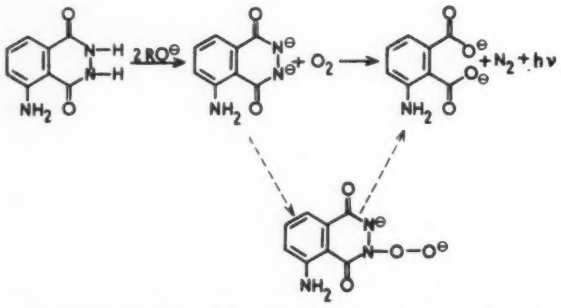


FIG. 6. The chemiluminescence of luminol in dimethyl sulfoxide.

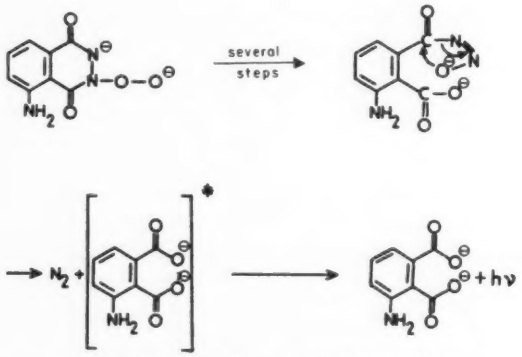


FIG. 7. A possible path for the formation of nitrogen.

inosinic acid derivative of oxyluciferin, L-IMP, is also an extremely potent inhibitor, while the IMP derivative of LH_2 is not effective for light production.

One of the interesting changes in the properties of oxyluciferin in the enzymatic reaction with ATP is the tremendous decrease in the intensity of fluorescence. Normally, the fluorescence of oxyluciferin excited at 348 mμ gives a strong emission peak at 544 millimicrons. However, when ATP and enzyme are added to such a reaction mixture the fluorescence intensity of E-L-AMP at this wave length decreases dramatically.

EMISSION SPECTRA

Figure 9 shows the emission spectrum of the firefly light reaction *in vitro* in glycyl-glycine buffer at pH 7.6. The peak emission for the bioluminescence is 562 mμ with the band ranging from below 500 mμ to above 630 mμ. Certainly as an absolute minimum the energy requirement for the bioluminescence should therefore exceed 57 Kcal/mole.

One might expect that some intermediate would occur in the light-emitting step which would have fluorescence properties similar to the bioluminescence emission and which could possibly be identified as the light-emitting species. For this reason careful measurements were made of the absorption and emission properties of a number of luciferin and oxyluciferin derivatives. At neutral pH or below luciferin shows an absorption peak at 327 mμ which corresponds to the activation peak for its fluorescence. When the pH is made higher than 9 the absorption peak shifts from 327 to 381 mμ.

The absorption spectrum of oxyluciferin (348 mμ at neutral pH) shows the same general shape and shift with pH as that of luciferin. The shift of approximately 20 mμ to longer wave length, as we go from the dihydro form, LH_2, to the oxidized form, L, is what one might expect from the addition of a double bond to the aromatic structure. The retention of both absorption bands with the same relative absorbances would also indicate that the only difference between the reduced and oxidized forms is the loss of two protons.

The fluorescence emission peak of luciferin is at 535 mμ and for oxyluciferin, 544 mμ. It is of interest that although the absorption peak of oxyluciferin shifts radically with changes in pH from acid to base the fluorescence emission remains at 544 mμ although altering in intensity. This would indicate that a separate portion of the molecule is responsible for the absorption as contrasted with emission. Thus the intramolecular energy transfer is dependent upon the ionic species present which in turn is a function of pH or, in the bioluminescence case, upon the type of hydrogen binding to the enzyme surface. This intramolecular energy transfer efficiency is reflected in the fluorescence yield of the luciferin and oxyluciferin molecules. The data presented in figure 10 indicate that both luciferin and oxyluciferin have the same ionizable groups; a carboxyl group showing a pK between pH 3 and 4 and a phenolic OH group with a pK at 8.4. The carboxyl group has been confirmed on the basis of infra-red spectra and other chemical tests. The fluorescence yield exhibits the most dramatic change depending upon the ionization of the hydroxyl group and it is most likely this group which is prevented from ionizing when oxyluciferin is bound on the enzyme surface, since the fluorescence of L-AMP relative to L shows the same relative decreases in intensity.

In none of the fluorescence measurements of luciferin and oxyluciferin under the various conditions described have we observed a fluorescence emission which matches the bioluminescence emission spectrum. However, as the results in figure 11 indicate, the adenylic acid derivatives of luciferin and oxyluciferin have radically different

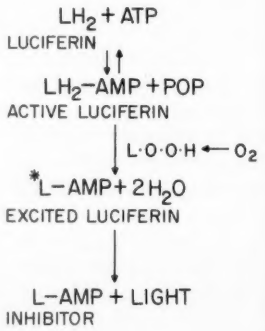


FIG. 8. Reactions of firefly luciferin in light production.

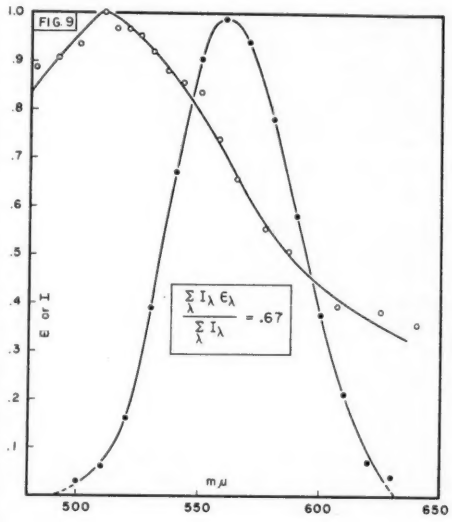


FIG. 9. Emission spectrum of firefly bioluminescence in glycylglycine buffer at pH 7.6. The peak emission is 562 mμ. Superimposed upon this spectrum is the normalized product $\epsilon(\lambda)$ A, the phototube spectral sensitivity.

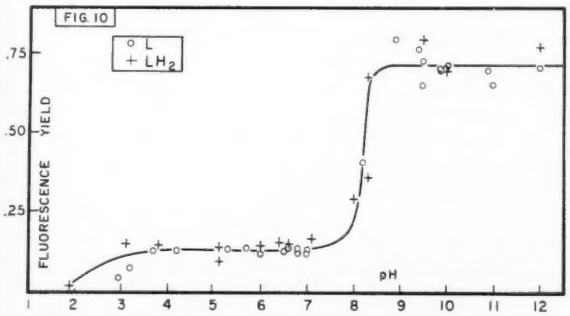


FIG. 10. Fluorescence quantum yields of firefly luciferin and oxyluciferin as functions of pH.

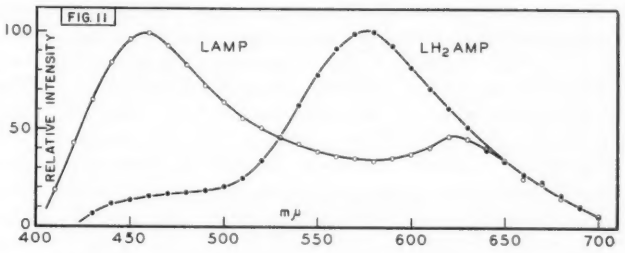


FIG. 11. Fluorescence of luciferyl adenylate and oxyluciferyl adenylate in acid solution.

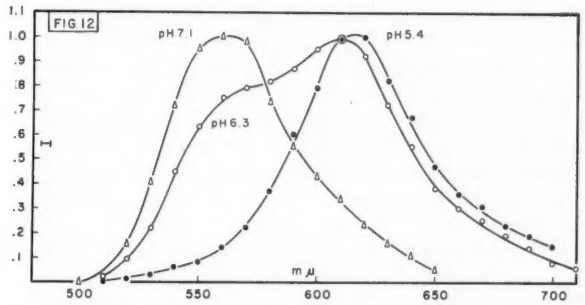


FIG. 12. Bioluminescence emission spectra for various pH values in glycylglycine buffer solution.

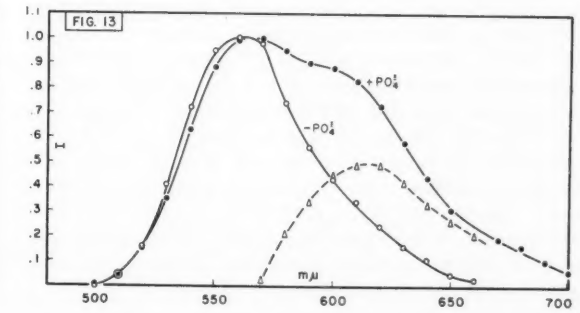


FIG. 13. Bioluminescence emission spectra at neutral pH with and without phosphate buffer.

fluorescence emission peaks. LH_2-AMP acid fluorescence has an emission peak at 570 mμ which corresponds closely to the bioluminescence emission curve. On the other hand, oxyluciferyl-adenylate fluoresces with a major peak around 460 mμ and a secondary minor peak at around 625 mμ. We shall discuss these data in connection with the mechanism of the light-emitting reaction in a later section.

QUANTUM YIELD OF FIREFLY BIOLUMINESCENCE

In considering the mechanism of the firefly light-emitting system it was essential that we have some understanding of the number of light quanta emitted per luciferin molecule used. The total light emitted during the enzymatic oxidation of luciferin over the range 10^{-12} mole to 10^{-10} mole was measured, and from these data we conclude that one light quantum is emitted per luciferin molecule oxidized (4).

This 100% efficiency for light emission is indicative of the high degree of protection afforded to the activated substrate molecule by the enzyme. That the tight binding of the LH_2-AMP or L-AMP to the enzyme is an important factor in determining the quantum yield is also suggested from temperature measurements. The quantum yield has been determined at 3° and at 27°C with no observed change in efficiency over this temperature range.

As the pH of the firefly solution is reduced toward acid it can be observed that the yellow green light intensity decreases tremendously leaving a dull brick orange glow. This variation in bioluminescence emission with pH is shown in figure 12 for three different pH values. At neutral (and alkaline) pH there is a single

emission band in the yellow-green region. At intermediate pH a red emission band appears at 616 mμ and at pH values below 5.5 the yellow-green emission is completely suppressed and only the red band is evident. The quantum yield has also been measured for various pH values. At acid pH the number of light quanta emitted per luciferin molecule oxidized is markedly lower than one indicating a predominantly dark reaction. However, at alkaline pH, although the rate of light emission is reduced to a small fraction of the rate at pH 7.6, the quantum yield is essentially unity. The change of quantum yield with pH corresponds rather closely in form to the fluorescence yield of luciferin and oxyluciferin at various pH's except for the fact that the pK has been essentially shifted one whole pH unit to the acid range for the bioluminescence reaction. This may represent the interaction of the enzyme with the phenolic OH group, altering in effect the fluorescence or chemiluminescence properties of the luciferyl-adenylate compounds.

One other factor has been shown to modify the emission spectrum of the *in vitro* bioluminescence reaction. We have indicated previously that inorganic phosphate is an inhibitor of light emission and the results shown in figure 13 indicate that phosphate produces a condition even at neutral or alkaline pH which is essentially similar to a reduction of the pH of the solution, that is, the appearance of the red emission band. The partial shift from the 562 mμ peak to the 616 mμ peak in the presence of phosphate or weak acid and the sharp decrease in quantum yield in acid pH are additional evidence that the enzyme is important in influencing the excited state. We feel that the important group on the luciferin molecules which influences the efficiency and light color is the phenolic OH group.

DISCUSSION OF MECHANISM

It would be appropriate at this time to summarize what is known of the mechanism of the bioluminescence and to point out some of the questions still unresolved. We know that during the enzymatic oxidation of luciferin, oxyluciferin is formed. On the basis of quantitative analysis the chemical formula of oxyluciferin differs from luciferin by only two hydrogen atoms. That oxyluciferin differs only slightly from luciferin is also evidenced by the striking similarity of their absorption, fluorescence and fluorescence yield curves and their respective variations with pH. This then is different, for example, from the chemiluminescence of luminol, where the release of N_2 from the amino phthalhydrazide molecule is sufficiently exergonic to leave the product amino phthalic acid molecule or an intermediate in an excited state from which blue light can be emitted. We have yet to resolve the steps by which the LH_2-AMP active intermediate, bound on the enzyme molecule, reacts with dissolved molecular oxygen to produce L-AMP and a light quantum. From the kinetic data on the fluorescence of E-L-AMP + PP $\rightleftarrows$ E + L + ATP and from the flash height data as a function of enzyme concentration we can safely say that there is only one site for light emission on the enzyme surface. The quantum yield of unity also precludes the utilization of two luciferin radicals on an enzyme molecule.

The fact that the acid fluorescence of synthetically formed LH_2-AMP is close to the bioluminescence emission would imply as in the luminol fluorescence and chemiluminescence an energy transfer from an excited product to the LH_2-AMP molecule. In addition, the acid fluorescence of L-AMP shows no yellow-green peak but does have a red fluorescence. However, *a*) LH_2-AMP can also be shown at more strongly acid pH, to exhibit a red fluorescence, *b*) the bioluminescence, and most chemiluminescences, occur only at neutral or basic pH values so that acid pH may have little relation to the environment during chemiluminescence, *c*) energy transfer should be concentration dependent and the results of the flash height versus enzyme concentration data would therefore rule out as in the case of the luminol chemiluminescence, an intermolecular energy transfer.

These data eliminate the possibility that two LH_2-AMP molecules would interact with oxygen in a reaction analogous to the one proposed by Linschitz in which one reduced molecule (in our case LH_2-AMP) is regenerated. It was conceivable that the exergonic step was the creation of the excited state of LH_2-AMP whose emission would correspond to the observed fluorescence of this compound. Energy transfer to the oxidized product (L-AMP) under these conditions might have given rise to the observed red emission.

We must account for a minimum of approximately 60 Kcal/mole in the oxidation of a single LH_2-AMP molecule which is probably more than is available from the production of a peroxide, obtained from a simple balancing of the equation LH_2-AMP + O_2 $\rightarrow$ L-AMP + H_2O_2. We have not yet been able to isolate unambiguously the excited state of the product molecule from which the light quantum is emitted, although we know that its fluorescence yield must be practially 100%. It is possible that the oxygen activation leading to the formation of hydrogen peroxide and the formation of a double bond in the ring structure of oxyluciferin, contributing as much as 30 Kcal of resonance energy, could furnish sufficient energy. The difference in binding energy of L-AMP and LH_2-AMP may also contribute to the creation of the excited state. Further experimental evidence, particularly on the possible formation of peroxide radicals, is necessary before a detailed description of mechanism can be given.

BRIEF COMMENTS ON OTHER BIOLUMINESCENT REACTIONS

A great deal of physiological and biochemical evidence is available for other bioluminescent reactions, unfortunately however very little can be said about the mechanisms involved (2). The use of the symbols LH_2 and E in the following discussion does not imply any similarity in structure but refers to an oxidizable heat-

stable substrate and a heat-labile enzyme or group of enzymes respectively.

Bacteria. Extensive studies by Strehler, Cormier, Totter, McElroy, Hastings and associates have shown the following reaction to be important for light emission:

$$FMNH_2 + RCHO + O_2 + E \rightarrow \text{light } (495\ m\mu)$$

The chain length of the aldehyde can vary from C_6 to C_{18} although the longer, even numbered aldehydes are more effective.

The following points are important in considering the mechanism of bacterial luminescence: *1*) The peak of the emission spectrum of bacterial light (*A. fischeri*) is 495 mμ. This is considerably removed from the yellow green fluorescence (530 mμ) of riboflavin or FMN. *2*) Kinetic data indicates that two reduced FMN molecules may be required. *3*) The data demonstrate that total light emission is dependent upon the amount of aldehyde added. The utilization of aldehyde occurs rapidly only under conditions which favor light emission. *4*) Thermodynamically, at least 58 Kcal/mole are required to obtain an excited state which would give the peak emission. Since light quanta at the wavelength of 420 mμ are also observed, the energetic requirement must be as high as 68 Kcal/mole. *5*) Added hydrogen peroxide to the complete enzyme system does not stimulate nor does catalase inhibit.

From such considerations McElroy and Green suggested the possibility that a peroxidation of an aldehyde molecule was the source of the excitation energy. They suggested that one reduced FMN combined with the aldehyde and that the second $FMNH_2$ combined with oxygen to form an organic peroxide. The presumption was that the organic peroxide acts as an oxidant for the aldehyde-$FMNH_2$ compound to give a highly excited molecule which would emit light. Although this proposal is in keeping with the observed facts, there is no direct chemical evidence to support it. It is possible, as argued by Strehler, that the utilization of aldehyde represents dark reactions and that such dark reactions occur only under those conditions which support luminescence. Unfortunately, until one can obtain a system which gives a quantum yield of one, as in the case of the firefly, this hypothesis is difficult if not impossible to eliminate.

Cypridina hilgendorfii—a luminous crustacean

$$LH_2 + O_2 + E \rightarrow \text{light } (460\ m\mu)$$

Unfortunately, the lack of highly purified luciferin (LH_2) and its dark auto-oxidation in the presence of oxygen has prevented a careful study of the fluorescent intermediates and products of the light reaction. The extensive chemical and physiological data on this system are reviewed by Harvey.

Odontosyllis—a polychaete worm

$$LH_2 + O_2 + E \rightarrow \text{light } (510\ m\mu)$$

The luciferin has been partially purified and its fluorescence emission is identical to the chemiluminescence emission (unpublished). Like cypridina it is readily dark-oxidized by molecular oxygen.

Pholas dactylus—a luminous clam

$$DPNH + FMN + O_2 + E \rightarrow \text{light}$$

This system appears to be very much like the bacterial system. However it has not been definitely established that an aldehyde is required nor that $FMNH_2$ can replace DPNH and FMN.

Omphali flavida—a luminous fungus

$$DPNH + X + O_2 + E \rightarrow \text{light } (530\ m\mu)$$

Although Airth and McElroy have demonstrated that DPNH can restore light emission in crude extracts it has not been possible to show a flavin or an aldehyde requirement. Some unknown substance is necessary for light emission and is gradually consumed.

Renilla reniformis—a sea pansy

$$LH_2 + AMP + O_2 + E \rightarrow \text{light}$$

Studies by Cormier have indicated that this system has some similarities to the firefly reaction but present evidence implies something quite different. Firefly luciferin will not replace *Renilla luciferin* in the above reaction.

Gonyaulax polyhedra—a luminous protozoan

$$LH_2 + E + O_2 \rightarrow \text{light}$$

Very little is known about the chemistry of light emission of this dinoflagellate which shows the interesting diurnal rhythm of luminescence (Hastings and Sweeney).

Apogon—a luminous fish

$$LH_2 + E + O_2 \rightarrow \text{light } (460\ m\mu)$$

This system appears to be identical to the *Cypridina* reaction (Johnson and Haneda). This represents one of the few examples in which the luciferins and luciferases of two unrelated species will cross react. The luciferins appear to be identical and only small differences can be found in the luciferases.

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Biosynthesis of diaminopimelic acid[1]

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TWELVE YEARS AGO Elizabeth and Thomas Work wrote "The Basis of Chemotherapy." Throughout the book there was the recurring theme of the desirability of a rational approach to chemotherapy. It was probably in pursuit of that ideal that Elizabeth Work began an investigation of the compounds which occur in bacterial organisms to determine if these were in any way different from those that occur in mammalian cells. If there were metabolic differences, exploiting these differences would indeed provide a rational basis for designing antibacterial agents. Her efforts were rewarded with success when she observed in two-dimensional paper chromatograms of a hydrolysate of *Corynebacterium diphtheriae* a ninhydrin positive spot that could not be detected when using hydrolysates of mammalian tissues. Work was able to isolate and characterize this new amino acid (19). The substance was identified as α-ϵ diaminopimelic acid (DAP). There are three possible stereoisomers of this structure (fig. 1). It was the meso isomer which was found in the *Corynebacterium*.

Work then systematically examined some 150 bacterial species in order to determine whether DAP was a chemical oddity relegated to *Corynebacterium* or whether the substance was more widely distributed. The results of that survey are summarized in table 1 (20). DAP was found to be generally present in all of the bacteria except for the gram-positive cocci. Outside of the bacteria DAP was found only in the blue-green algae though recently there has been a report of its occurrence in a green alga (10, 12). The finding of a unique compound common to the bacteria and blue-green algae is particularly interesting in view of Ferdinand Cohn's toxonomic classification of the 1880's. Cohn placed the blue-green algae with the bacteria since they also lack a discrete nucleus and they do not have the sexual means of reproduction present in the other algae.

Among other organisms DAP has not been detected. This list includes red, brown and most green algae, fungi, protozoa, yeasts, plant viruses, mammalian tissues and so on. This is indeed a remarkable distribution of a substance to be so generally present in one group of organisms and so thoroughly absent in all other groups.

Shortly after these observations the work of Holdsforth (11) and Cummins and Harris (5) demonstrated that not only did DAP enjoy a unique distribution intercellularly, but within the cell as well. For, though DAP is an amino acid, it was not in the bacterial protein that the compound was found but rather it turned out to be an important component of the bacterial cell wall. It was also established by B. D. Davis (6) and by Work (7) that DAP functions not only as a bacterial constituent but also as an intermediary in the biosynthesis of lysine. However, again, this is only so for the bacteria and possibly blue-green algae. In other organisms, that can synthesize lysine, the pathway involves 6 carbon compounds such as α-amino adipic acid. This diversity in lysine biosynthesis represents one of the best-documented examples of an exception to the unity of biochemistry.

The problem that has enagaged the attention of our laboratory is the mode of biosynthesis of DAP. Figure 2 summarizes some of the earlier information in this area. *E. coli* mutant 26-26, isolated in B. D. Davis' laboratory, differed from the wild type in manifesting a requirement for lysine. This mutant lacks the decarboxylase that converts meso-diaminopimelic acid to L-lysine and this is responsible for its lysine requirement (6, 7). In addition, M-26-26 was found to excrete meso-DAP, and, most peculiarly, to excrete L-DAP as well. This discovery by Wright and Cresson (21) was unexpected since, as in the case of *C. diphtheriae*, *E. coli* contains only the meso isomer in its cell wall and the decarboxylase

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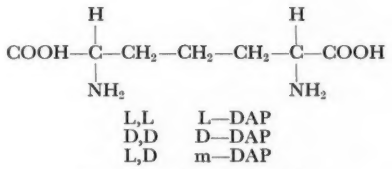


FIG. 1. Diaminopimelic Acid.

TABLE 1. *Distribution of DAP (Data of E. Work)*

Present	Absent
Eubacteriales	Micrococcaceae
Lactobacillus	Streptococcus
Propionibacterium	Diplococcus
Corynebacteriaceae	Other algae
Bacillaceae	Fungi
Mycobacteria	Protozoa
Mycelial actinomyces	Yeasts
Myxobacteria	Plant viruses
Blue-green algae	

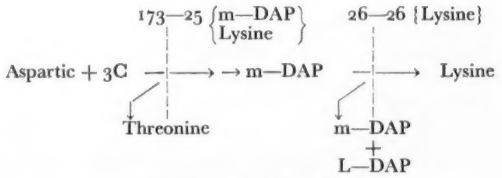


FIG. 2. Lysine biosynthesis.

is specific for the meso isomer. Antia *et al.* (1) established that *E. coli* contains a racemase that interconverts meso-DAP and L-DAP and this, at least, partially explains why L-DAP should appear in the medium.

Mutant 173-25 is presumably blocked early in DAP synthesis. Nutritionally it shows a relative requirement for lysine and an absolute requirement for DAP (6). This mutant excretes threonine, but, as it was subsequently established by the Carnegie group (13) that threonine cannot be a precursor of DAP, this finding does not provide a clue to the pathway of biosynthesis of DAP.

Using the isotope competition method the Carnegie group was able to establish aspartic acid as a precursor of four of the seven carbon atoms of diaminopimelic acid (13). The source of the other three carbon atoms was unknown.

In order to gain some insight into the nature of the 3 carbon precursor resting cells of M-26-26 were used with the rational that any compound that would augment the excretion of DAP in the presence of aspartic acid might be a source of the three carbon compound. A very effective combination of carbon compounds that would facilitate DAP excretion was aspartic and pyruvic acids (8). Provided with this information as to carbon sources, it was then possible to obtain a cell-free system capable of carrying out the synthesis of DAP from its early precursors. Once again M-26-26 was used. As its extracts do not contain DAP decarboxylase any DAP formed in such extracts will not be further metabolized.

All of the enzymes that participate in DAP synthesis in *E. coli* are stable and easily obtained in cell-free condition. Table 2 lists the components of the system that have been shown to be necessary for this synthesis (8). This includes Mg^{++}, ATP, DPN, TPN and glutamate in addition to aspartate and pyruvate as carbon sources. This is certainly not a complete compilation of the substances required for DAP synthesis. From what follows it will be apparent that pyridoxal phosphate, coenzyme A and Co^{++} must also be required. As yet this has not been demonstrated in the overall system. In this system all of the intermediates and enzymes in which one is interested play an interacting role in DAP synthesis. This initial extract can therefore serve as an assured source for any DAP synthesizing enzyme and it can also be used as a means of testing possible intermediates superior to the intact cell since in the enzyme system one need not be concerned with considerations of cellular permeability.

In an effort to gain an insight into the nature of possible intermediates additional DAP requiring mutants were sought that might accumulate compounds in the medium more informative than in the case of 173-25. Through use of the penicillin technique a new DAP auxotroph (D-1) was obtained. It has been possible to isolate and characterize two new compounds accumulated by D-1 in the medium in which it has been grown. The structure of the first one to be isolated is shown in figure 3. It is a succinylated derivative of DAP, one of the amino groups being blocked by an acyl linkage with succinic acid. The DAP moiety of this compound is the L-isomer (9).

It was conceivable that this compound accumulated in the medium as some sort of detoxified product of an intermediate. If this were so then, unlike the true intermediate, it should be inactive as a precursor of DAP.

TABLE 2. *DAP Enzyme System*

	μM/Ml	Dependency
MG^{++}	10.0	2.3
ATP	10.0	3.3
DPN	0.15	2.0
TPN	0.15	6.1
Glutamate	2.0	4.5
Aspartate	25.0	120.0
Pyruvate	20.0	3.3
(7.0) Imidazole	100.0	

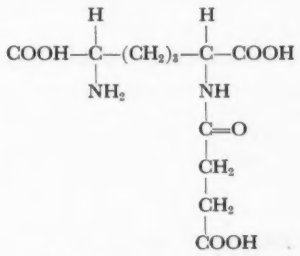


FIG. 3. Structure of intermediate.

However when N-succinyl-L-DAP is added to the overall enzyme system it is transformed to DAP. The enzyme responsible for this transformation is a deacylase. It catalyzes the following reaction:

N-succinyl-L-diaminopimelate → L-diaminopimelate + succinate

This enzyme is absent in D-1.

The L-configuration of the DAP moiety and the fact that L-DAP is the product of the deacylase enables one to decide between the possibilities which were a consequence of the finding that M-26-26 excreted both the meso and L-forms of DAP.

Path 1 → M-DAP → L-Lysine
↓ ↑
L-DAP
Path 2 → L-DAP ⇄ M-DAP → L-Lysine

Clearly the choice must go to path 2. Thus the racemase which interconverts meso and L-DAP is an obligate biosynthetic enzyme.

To gain further assurance that the deacylase, as well as N-succinyl-L-DAP are involved in DAP synthesis, Dr. Kindler undertook a study of the enzyme. It has been purified 35-fold and a co-factor requirement for a metal has been established. Cobalt is most effective though Mn^{++} and Zn^{++} are somewhat active. The distribution of the enzyme among bacterial species was examined. The results of that survey are presented in table 3. Bacterial species that contain DAP in their cell wall or utilize DAP for lysine synthesis were found to possess the deacylase.

In the course of isolating N-succinyl-L-DAP it was observed that an additional compound was accumulated by D-1. This compound was isolated and identified as N-succinyl-α-amino-ε-ketopimelic acid, i.e., the keto analogue of N-succinyl-L-DAP (fig. 4).

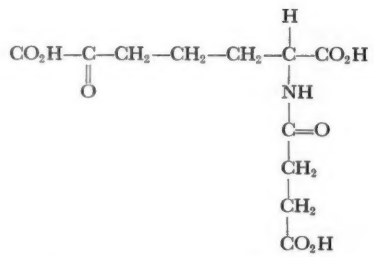
There are several aspects of the structure of the keto compound that are satisfying. Its structure supports the notion of a condensation between pyruvate and some form of aspartic acid. In addition, it provides a rationalization for the succinate acting as a blocking group. If the succinate were removed the molecule would cyclize to the corresponding piperidine derivative.

When the keto compound was incubated in the overall enzyme system it, too, was transformed to DAP. The enzyme that makes this conversion possible is a transaminase that catalyzes the following reaction:

N-succinyl-α-amino-ε-keto-pimelate + L-glutamate ⇄ N-succinyl-L-diaminopimelate + α-ketoglutarate

TABLE 3. *Distribution of Succinyl-DAP Deacylase*

Organism	DAP in Cell Wall	S. A. μM/Min/Mg
E. coli	+	0.096
A. vinelandii	+	0.074
C. diphtheriae	+	0.063
R. spheroides	+	0.037
A. faecalis	+	0.035
B. cereus	+	0.028
M. lysodeikticus		0.059
M. lactolyticus		$<10^{-3}$
L. casei		$<10^{-3}$
S. hemolyticus		$<10^{-3}$



N-succinyl-α-amino-ε-keto-pimelate

FIG. 4

TABLE 4. *Distribution of SD Transaminase*

Organism	Cell Wall Component	Lysine Requirement	Activity μM/Min/Mg × 10^{-3}
Alc. faecalis	M-DAP	−	98.9
E. coli	M-DAP	−	47.7
M. lysodeikticus	Lysine	−	25.2
L. arabinosus	M-DAP	+	1.4
L. casei	Lysine	+	0
Strep. C203S	Lysine	+	0
Brewer's yeast		−	0
Pig heart extract		+	0

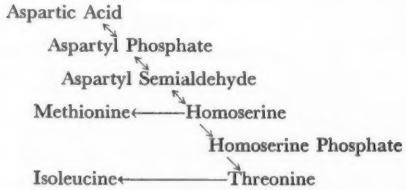


FIG. 5. The aspartic acid family.

This enzyme has been purified by Mrs. Peterkofsky 140-fold. A pyridoxal phosphate requirement for the enzyme has been demonstrated and it has been shown to be very specific for its substrates. The enzyme is distinct from the other transaminases which Rudman and Meister reported in *E. coli* (14).

When the transaminase distribution was surveyed (table 4), the same pattern was found as was the case for the deacylase. In organisms, such as *A. faecalis* or *E. coli*, that have DAP in their cell wall and synthesize lysine, there is a good deal of this enzyme. *M. lysodeikticus*, which does not have DAP in its cell wall but synthesizes lysine, and *L. arabinosus*, which does have DAP in its wall but depends on an exogenous supply of lysine, also contain the transaminase. However, *L. casei* or the streptococci which neither contain DAP nor use it as an intermediary in lysine synthesis are devoid of the transaminase.

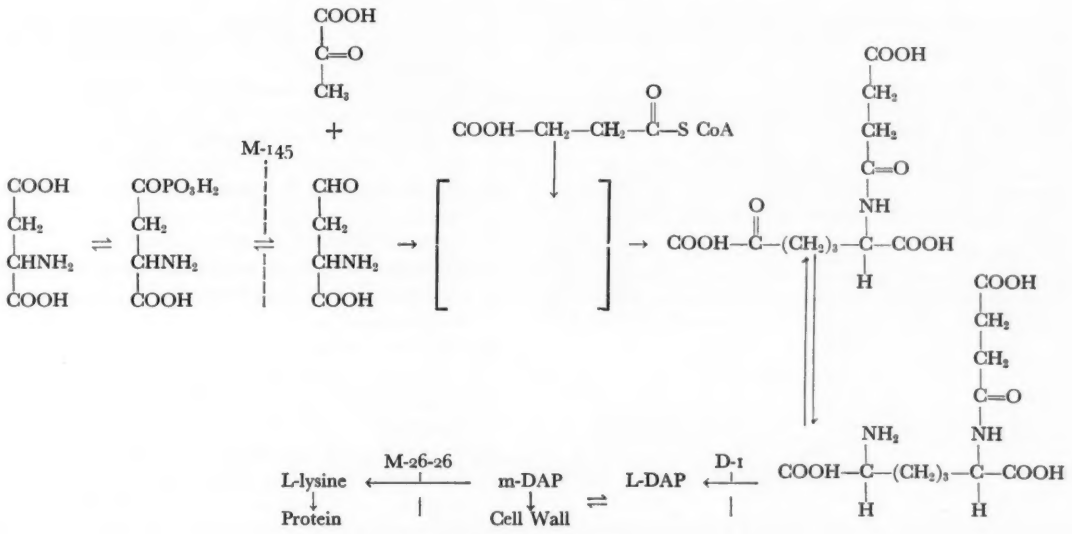


FIG. 6. Biosynthesis of lysine.

This parallelism in the occurrence of DAP and the transaminase provides strong support for the biosynthetic role of the keto compound.

Recently attention has been turned to the earlier steps in the biosynthesis. Figure 5 schematizes the pathway from aspartic acid to threonine that has been revealed by the work of Black and Wright (2–4) and Watanabe, Shimura and Konishi (15–18). Aspartic acid is converted to aspartyl phosphate. This compound is reduced to aspartyl semialdehyde which, in turn, is reduced to homoserine. Another kinase converts homoserine to homoserine phosphate which is then isomerized to threonine. In addition, the fact that homoserine is the source of 4 methionine carbons and threonine is utilized in isoleucine synthesis is also indicated in figure 5. Since aspartic acid is the precursor of four of the carbon atoms of DAP the question arises: at which of these intermediates is the branch point which leads to DAP and lysine? The choice of possible precursors can be narrowed since isotope experiments by the Carnegie group have eliminated homoserine and threonine. The decision must be made among aspartic acid itself or aspartyl phosphate or aspartyl semialdehyde. Attempts to settle this question in the overall enzyme system by adding any one of these three compounds as sources of the four carbons would be fraught with dangers of interpretation in a system involving reversible enzymes that are present in large amounts relative to the overall rate of synthesis. Presumably, any one of these compounds could serve as carbon source even though they were not directly involved in the synthesis.

Once again mutant methodology has been helpful in the solution of this problem. A mutant was sought which was blocked at one of the early reactions in threonine synthesis. M-145 was isolated on the basis of its multiple nutritional requirements for members of the aspartic acid family of amino acids. The organism has an absolute requirement for DAP, methionine and threonine and relative requirements for lysine and isoleucine. Analyses of extracts prepared from this mutant indicate a complete absence of aspartyl semialdehyde dehydrogenase. The inability to convert aspartyl phosphate to aspartyl semialdehyde would result in a DAP and lysine requirement in addition to those for methionine, threonine and isoleucine, only if the branch point leading to DAP and lysine was located at aspartyl semialdehyde. Further experiments that support this point are the observations that, though extracts of M-145 cannot carry out the overall synthesis of DAP starting from aspartic acid, they can form DAP from homoserine. In such a situation, presumably, the back reaction of homoserine dehydrogenase is being used for aspartyl semialdehyde formation. In addition, supplementing M-145 with an aspartyl semialdehyde dehydrogenase preparation purified from yeast confers on these extracts the ability to make DAP from aspartate.

These findings make it possible to incorporate aspartyl phosphate and aspartyl semialdehyde and the two enzymes which form the semialdehyde from aspartic acid into the scheme of lysine biosynthesis summarized below (fig. 6).

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Oxidative phosphorylation in submitochondrial systems

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The mechanism of oxidative phosphorylation still remains one of the major challenges to the enzyme chemist. The association of the enzymes of electron transport and phosphorylation in 'solid-state' lipoprotein arrays in the mitochondrial membranes presents formidable experimental problems in attempts to satisfy the three classical requirements for elucidation of the nature of a multienzyme system: *a*) isolation of the individual enzymes, *b*) identification of the individual reactions catalyzed, and *c*) *in vitro* reconstruction of the system from known components. Recent work is beginning to yield some identifiable fragments of this complex mechanism, offering considerable hope that the chemical events in respiratory energy transduction may be successfully reconstituted with components of known enzymatic capability.

This paper cannot cover all phases of oxidative phosphorylation, but is limited to recent progress in the dissociation and identification of individual reaction steps of respiratory chain phosphorylation as well as current efforts to reconstitute the coupling mechanism in submitochondrial systems.

Mitochondrial structure and experimental approaches to phosphorylation mechanisms. It is now well established that the respiratory carriers are present in or on the mitochondrial membrane(s) (cf. 2, 31, 52). With the possible exception of cytochrome c, which is easily detached, the carriers are tightly bound to the lipoprotein structure. The enzymes necessary for energy coupling seem also to be bound to the membranes, since they remain attached after treatment with digitonin (26, 41, 52) or sonic radiation (39, 57).

The association of the catalysts of respiration and coupled phosphorylation with the insoluble lipoprotein membranes presents three main problems: first, the probability that respiration and energy coupling occur by an extended sequence of interactions between large (protein) molecules, or between specific active groups attached to large protein molecules, without the intervention of free-moving, dialyzable low-molecular weight intermediates or 'shuttle' molecules (15, 41, 61). There may be a biological necessity for structural organization of these catalysts in a moderately rigid, geometrically organized constellation in the membrane, to minimize the path distance between slowly diffusing large molecules and to maximize probability of interaction. This fact thus will make it necessary to identify the types of reaction occurring at 'active sites' of these proteins before the mechanism of oxidative phosphorylation can really be understood; the active sites of these enzymes become in effect the 'substrates' or 'intermediates' in oxidative phosphorylation. Secondly, new approaches will have to be developed in the technology of solubilization and purification of individual homogeneous enzyme molecules from the lipoprotein structure. Some rule of thumb experimental approaches to separation and purification of 'insoluble' enzymes have already been developed, including use of cholate and other detergents, solvents,

such as alcohols, and proteolytic enzymes. However these are only partially successful and may have to be replaced by more rational approaches based on better knowledge of protein and lipid structure as well as the ultra-structure of the mitochondrial membrane itself. Finally, the possibility that intermediate reactions of respiration and phosphorylation may take place in regions where the chemical potential of water molecules is low (21) (i.e. in local nonaqueous or lipid phases (32)) may require some new concepts in *in vitro* experimentation or reconstruction. The requirement of certain neutral lipid fractions for reconstitution of segments of the respiratory chain (cf. 32), although not yet convincingly specific, illustrates some of the problems to be met.

While the association of the energy-conserving reactions of the respiratory chain in the mitochondrial membrane may be regarded as a formidable technical problem, on the other hand it may provide important clues as to the molecular structure and cellular functions of membranes in general. Furthermore, recent work on the morphology and the respiration-activated swelling and contraction of mitochondria may also provide important leads into the mechanism of oxidative phosphorylation, as will be developed below.

The respiratory carriers and coupling enzymes appear to exist as organized 'assemblies' in the membranes, in which the carriers are present in simple finite molar ratios, as indicated by difference spectroscopy (15) and chemical and enzymatic analysis of fragments of mitochondrial membranes (2, 26, 28, 31, 41, 52). Calculations based on difference spectra, the nitrogen content of mitochondria, and mitochondrial counts and volume indicate that a single rat liver mitochondrion may contain at least 5000–10,000 sets of respiratory 'assemblies', possibly more depending on the necessary assumptions (41, 42). Fragmentation and centrifugal studies indicate these assemblies to be distributed fairly evenly over the membrane, presumably the inner membrane (26, 28, 52). Such calculations also suggest that a large fraction of the protein mass of the membrane may be made up of these carrier assemblies; 40% is a not unreasonable estimate, and it could be still higher (cf. 2). More detailed analysis is limited by the currently changing views on the structure of the outer and inner mitochondrial membranes (cf. 69) and the manner of stratification of lipid and protein molecules. These considerations, however, show the mitochondrial membranes to be more than passive limiting structures; they may more appropriately be thought of as complex multienzyme systems (41). Changes in permeability and physical state of the membranes could be expressions of changes in the condition or function of the respiratory catalysts in the membranes; this thesis is developed below and may provide important approaches to the mechanochemistry of the coupling enzymes.

The respiratory chain. It has been demonstrated that there are three energy-conserving sites in the respiratory chain between DPN and oxygen in rat liver mitochondria (43). The claim by Dallam and Howard (25) that a fourth site may be evoked by thyroxine requires confirmation. Two sites lie between DPN and cytochrome c (6) and the third between cytochrome c and oxygen (60).

TYPE I General case (Slater, (19))

$$AH_2 + B + C \rightleftarrows A \sim C + BH_2$$
$$A \sim C + ADP + P_i \rightleftarrows A + C + ATP$$

Sum: $AH_2 + B + ADP + P_i \rightleftarrows A + BH_2 + ATP$

Specific case (Lehninger, 1954, cf. (30))

$$DPNH + HS - E \rightleftarrows DPNH - S - E$$
$$DPNH - S - E + FAD \rightleftarrows DPN^+ \sim S - E + FADH_2$$
$$DPN^+ \sim S - E + P_i \rightleftarrows DPN^+ + P \sim S - E$$
$$P \sim S - E + ADP \rightleftarrows HS - E + ATP$$

Sum: $DPNH + FAD + P_i + ADP \rightleftarrows DPN^+ + FADH_2 + ATP$

TYPE II Lipmann (31), modified by Slater (19).

$$AH_2 + P_i \rightleftarrows AH_2P_i$$
$$AH_2P_i + B \rightleftarrows A \sim P + BH_2$$
$$A \sim P + ADP \rightleftarrows A + ATP$$

Sum: $AH_2 + B + ADP + P_i \rightleftarrows A + BH_2 + ATP$

FIG. 1. Postulated energy-coupling mechanisms.

Thermodynamic considerations, as well as experimental test of known segments of the chain (cf. 72) suggest the first energy-conservation step to lie between DPN and flavoprotein, the second between cytochrome b and cytochrome c, and the third between cytochrome c and oxygen as shown. Application of the spectrophotometric 'cross-over' approach (15) also suggests approximately these locations, but this method suffers from ambiguities in distinguishing between 'inhibited' sites and energy-conserving sites (cf. 14, 16). However, other locations of the energy-conservation mechanisms have also been postulated such as vitamin K (1, 3, 8–11, 58, 82, 83) flavoprotein (29, 30, 56) and coenzyme Q (35).

Further progress in identifying the exact loci of the respiratory chain phosphorylations will have to await resolution of the precise number and sequence of the electron carriers in the normal phosphorylating respiratory chain.

Postulated mechanisms of oxidative phosphorylation. Brief consideration of postulated mechanisms or reaction patterns of oxidative phosphorylation is necessary as orientation before discussion of current research efforts. Many mechanisms have been proposed. However they may be reduced in their simplest terms to one of two general reaction patterns, as Slater has pointed out (72).

The first, designated the 'Type I mechanism' by Slater (72), was first postulated by Slater (73) and independently in more detail by Lehninger (44). In figure 1 it is seen that this mechanism invokes a molecular participant C which, on passage of electrons from Carrier A to Carrier B, forms a 'high-energy' bond with the oxidized form of Carrier A. The 'high-energy' compound $A \sim C$ formed then is postulated to react with phosphate and ADP to form ATP; the more specific treatment of Lehninger (44) is also shown. It is the distinguishing characteristic of Type I mechanisms that

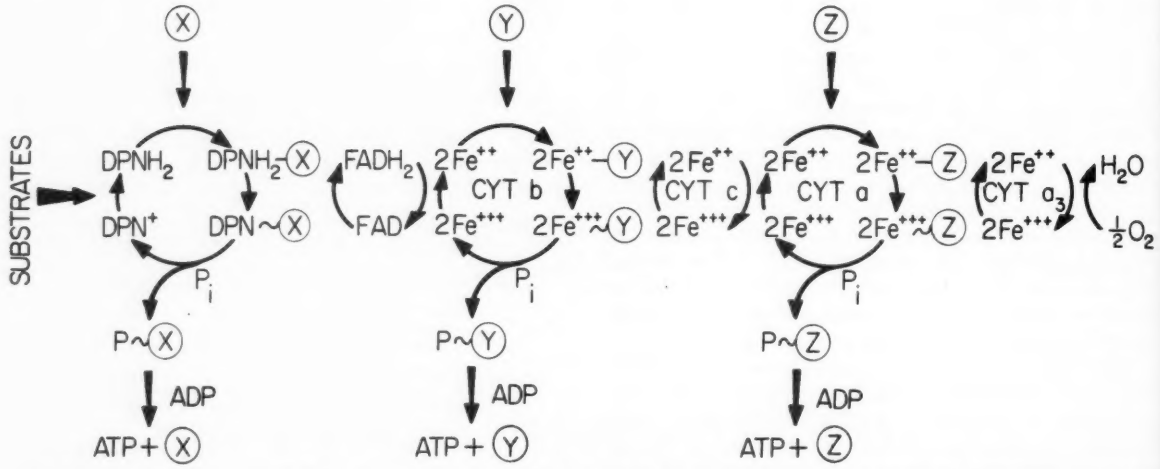


FIG. 2. Schematic representation of the energy-coupling at all three sites in the complete respiratory chain (52). This incorporates the Type I mechanism postulated by Lehninger *et al.* (cf. (52)). A more elaborate representation may be needed in view of the recent findings on 'M-factor.'

inorganic phosphate does not react with either of the carrier molecules directly. The mechanism postulated by Chance and Williams (15) also is a Type I mechanism.

Each of the three phosphorylation sites in the respiratory chain is postulated to have such a sequence of reactions; for example, the scheme in figure 2 shows a Type I representation of the complete phosphorylating respiratory chain (52).

In Type II mechanisms, first suggested by Lipmann (55), Hunter (37) and others, the reaction pattern is actually similar, but instead of the component C, inorganic phosphate itself is postulated to react directly with a carrier molecule to form a phosphorylated electron carrier molecule.

Available evidence favors Type I mechanisms. All the known 'substrate-level' phosphorylations (triose phosphate dehydrogenase and the pyruvic and α-ketoglutaric dehydrogenases) are clearly in this class, since 'high-energy' thioesters of the oxidized substrates are the first demonstrable products, which then undergo secondary displacement reactions with phosphate. Actually respiratory chain phosphorylation may have been derived, in an evolutionary sense, from the more primitive substrate-level phosphorylations associated with anaerobic fermentation cycles. The most important point of evidence however is that inorganic phosphate is not required for electron transport, either in aged systems in which coupling mechanisms are inactivated (5) or in systems in which phosphorylation is uncoupled with dinitrophenol (72, 74). Much recent speculation on mechanisms of phosphorylation (see below) involves Type II mechanisms and has ignored this important fact. On the other hand it could perhaps be maintained that trace amounts of inorganic phosphate suffice for maximum rates of electron transport.

It should be mentioned that energy transfer between two chemical reactions can be effected not only by 'coupling' through a common intermediate, as in the above mechanisms (other formulations on this principle are possible) but could also occur through fluorescence phenomena, resonance coupling, through charge-transfer complexes or by free radical mechanisms (27). However, as yet it has not seemed necessary to postulate mechanisms other than those involving the classical common intermediate to explain available data, however attractive speculation on other molecular energy-transfer devices may appear.

Two main routes to the mechanism of the energy-coupling reactions are being followed in current work with submitochondrial systems. The first is the seemingly more direct approach, namely, attempts to identify the specific energy-coupled carriers and the chemical species of such a carrier which represents the first form in which energy of electron transfer is conserved. The second route to the mechanism of coupling is an indirect one—the 'back-door approach,' which exploits the fact that the terminal or perhaps all of the reactions of the coupling mechanism (i.e. those summarized in reaction 2 of Type I mechanisms) are reversible, permitting the possibility that the individual reaction steps may be isolated and identified by proceeding from the known end-product ATP step by step, back toward the carrier level.

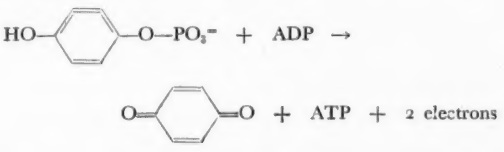
Submitochondrial phosphorylating systems. The observed occurrence of oxidative phosphorylation in particles or extracts derived from mitochondria, with far less structural and enzymatic organization than shown by intact mitochondria, has opened the possibility of dissociation and reconstruction. The pioneering work of Pinchot on phosphorylation in bacterial extracts (*A. faecalis*) (63; see also 36) has been followed by successful demonstrations of phosphorylation in extracts from yeast, *A. vinelandii* (70, 75) and *M. phlei* (8–11, 82) among other

microorganisms. It must be recalled that bacteria do not contain mitochondria; it appears probable that respiration and phosphorylation in bacteria may take place in the protoplast membrane (59).

The demonstration by Cooper and Lehninger (22) of oxidative phosphorylation in fragments of rat liver mitochondria derived by digitonin treatment was followed by reports of phosphorylation in sonic extracts of liver mitochondria (39, 57) and in alcohol-phosphate treated heart mitochondria (33).

Approaches to the mechanism at the electron carrier level. The real heart of the problem of oxidative phosphorylation is the chemical process by which oxidation-reduction energy is conserved during the exergonic electron transfers. As outlined above, three of the carriers in the chain are believed to form 'high-energy' intermediates by combination with either phosphate or with hypothetical substances variously designated as C,E,I, or X (cf. 72). By analogy with the role in energy-conservation of the —SH group in triose phosphate dehydrogenase (67, 68) certain functional groups of respiratory carriers, such as —SH groups (44), the amide of DPN, the carbonyl group of the iso-alloxazine ring, and the formyl group of cytochrome a + a_3 porphyrin have been suggested as participants in formation of such energy-conserving derivatives of the carriers during electron transport (29) but as yet no convincing evidence has been adduced supporting these suggestions.

Recent developments on the possible participation of reversible quinol-quinone systems in the phosphorylating respiratory chain, such as vitamin K (1, 3, 8–11, 58, 82, 83) and coenzyme Q (35), provide chemically attractive potentialities for a mechanism of energy-conservation. These conceptions have their basis in the hypothesis of Wessels (83) first proposed in 1954, and the chemical demonstrations in the laboratories of Todd (19) and Wieland (84) that oxidation of quinol monophosphates can lead to phosphate transfer to acceptor molecules, including H_2O and ADP (cf. 34).

(1) 

While the chemistry of such oxidative phosphate transfers from quinol phosphate is now quite soundly based, there are two fundamental shortcomings. One of these is the fact that no kinetically or thermodynamically satisfactory mechanism has been proposed for the original formation from inorganic phosphate of the quinol phosphate whose oxidation is necessary for phosphorylation of ADP.

A second shortcoming is the fact that these mechanisms are instances of Type II reactions, in which phosphate is postulated to react directly with the electron carrier, a feature which is not in accord with available evidence on the action of the respiratory chain.

It is not yet certain on kinetic grounds (13) that coenzyme Q is a normal member of the respiratory chain, although work in Green's laboratory clearly shows its ability to oxidize flavoprotein and to transfer electrons ultimately to cytochrome c, via or parallel to cytochrome b (cf. 32). Most current work on coenzyme Q is concerned with its place in the respiratory chain, and so far little direct work has been done on its action as a vehicle of phosphorylation, although Hatefi has noted some suggestive effects of phosphate on the action of coenzyme Q (35).

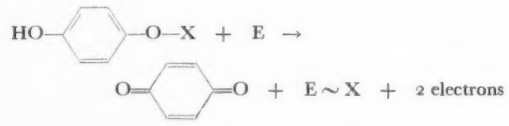
On the other hand, recent work on vitamin K suggests it to be involved in both respiration and phosphorylation. Respiration and phosphorylation in extracts of *Mycobacterium phlei* (8–11, 82) and in liver mitochondria (1, 3) are inactivated by irradiation in the ultraviolet and can be restored by addition of vitamin K_1. Perhaps the more interesting and revealing system at present is the *M. phlei* system of Brodie. He has found that respiration and phosphorylation in UV-inactivated extracts may be restored by addition of vitamin K_1 or by an endogenous naphthoquinone similar to vitamin K_1 which he has isolated from the organism, but not by vitamin K_3 (menadione). The latter, which lacks the side chain of vitamin K_1, can restore only oxidation. Coenzyme Q does not restore. More recently Brodie made a preliminary report of evidence that a phosphorylated derivative of vitamin K_1 (or possibly its cyclized 'chromanol' isomer (74a)) may be formed on anaerobic incubation with the *M. phlei* extract in the presence of labeled phosphate. After separation of this derivative it was shown to be active in transferring phosphate to ADP when incubated aerobically with the extract, although there appears to be a puzzling discrepancy in the specific activity of the ATP so formed (71). Observations of this type may yield important clues into the role of naphthoquinones in oxidative phosphorylation.

The direct interaction of phosphate with the quinol-quinone electron carrier systems, which is a feature of most current thinking and work on vitamin K and coenzyme Q, is contrary to known properties of the respiratory chain, as mentioned above. However it is possible that the facile tendency of coenzyme Q to isomerize to ubichromenol by forming a fused chromene ring with a 'loop' of isoprene from the side chain (40) may afford chemical potentialities for explaining nonphosphorylating and phosphorylating electron transport with a single quinone carrier. It is of interest that vitamin K_1 also apparently forms such a derivative (74a), the so-called naphthotocopherol.

Recently Chmielewska (20) has postulated mechanisms of both nonphosphorylating and phosphorylating electron transport through vitamin K_1; the former is suggested to occur through naphthoquinol-naphthoquinone oxido-reduction and the latter through cyclized derivatives of vitamin K_1. Such a picture would rationalize the fact that the quinones without side chains, such as vitamin K_3 (menadione), reactivate oxidation

but not phosphorylation in UV-irradiated *M. phlei* extracts, whereas the isoprenoid side-chain is required to reactivate phosphorylation as well as oxidation.

Another way, and perhaps the easiest way, to remove objections to the direct reaction of phosphate with a quinone carrier, which has apparently not been proposed before, is to postulate that some other nucleophile 'X' reacts directly with the quinol, rather than phosphate. Oxidative transfer of X from quinol-X to an acceptor E would then be followed by a displacement of X from E ~ X by phosphate. The E ~ P formed could then donate phosphate to ADP, as follows:

(2) 

(3a) $$E \sim X + Pi \rightleftharpoons X + P \sim E$$

(3b) $$P \sim E + ADP \rightleftharpoons ATP + E$$

The 'back-door' approach to the coupling mechanism. A second and independent entree to the mechanism of energy coupling is afforded by the finding that at least some of the intermediate coupling reactions are reversible. Thus by starting with ATP, the end-product of coupling, it appears feasible to work back in the coupling scheme by identifying intermediate steps. Several 'partial reactions' of oxidative phosphorylation have been identified which provide a basis for this approach. The first, of course, is the now well-known stimulation of hydrolysis of ATP in mitochondrial and submitochondrial systems by dinitrophenol and certain other uncoupling agents. In intact mitochondria this reaction is very prominent but complicated by the permeability properties of the membranes and the swelling-contraction phenomena described below. In digitonin particles from rat liver mitochondria the reaction has been studied intensively (23). It is specific for the 5′-triphosphate of adenosine, and is inhibited by ADP. The stimulation is given by dinitrophenol and related halophenols, by dicumarol, and gramicidin. It is inhibited by azide at 0.002 M. Addition of Mg^{++} stimulates the ATP-ase independently but this stimulation is not concerned in the coupling mechanism, since Mg^{++} is unable to stimulate the respiration of digitonin particles in the absence of ADP whereas DNP does (45). Wadkins (76) has shown that the ATP-ase is also stimulated by arsenate; such 'arsenolysis' of ATP is thus equivalent to that occurring in the triose phosphate system (cf. 72).

The second 'partial reaction' is the ATP-P_i^{32} exchange, first observed in intact mitochondria by Boyer *et al.* (7) and intensively studied in digitonin particles by Cooper and Lehninger (24). The rapid incorporation of inorganic phosphate labeled with P^{32} into the terminal phosphate of ATP requires the presence of ADP (24, 52) and it is specific for adenine nucleotides. This reaction is inhibited by DNP, dicumarol, gramicidin, and arsenate, as well as by azide and sucrose. The exchange rate and the ATP-ase activity in digitonin particles is maximum when the carriers are in the oxidized state (79) and strongly inhibited when the carriers are maintained in the reduced state, in the presence of β-hydroxybutyrate and succinate as reductants, respiration being blocked by anaerobiosis or by cyanide. These findings have now been fully confirmed in intact mitochondria by recent work in Slater's laboratory (17a), in contrast to earlier work by Myers and Slater which indicated no effect of oxidation-reduction state on ATP-ase activity (72). These findings thus eliminate the need to postulate an intermediate 'I' in the coupling sequence (cf. (72) for details).

The third partial reaction, which has provided perhaps the most important opening wedge, is the ATP-ADP exchange reaction. This was first observed in phosphorylating particles from *A. vinelandii* by Rose and Ochoa (70). However ATP-ADP exchanges are also catalyzed by enzymes not related to oxidative phosphorylation, such as the succinyl 'P-enzyme' (38), among others, and they are also brought about by adenylate kinase, which is present in mitochondria. However, the experiments of Cooper and Lehninger (24) first showed that ATP-ADP exchange in digitonin fragments is related to oxidative phosphorylation, by demonstrating that it is inhibited by DNP. The ATP-ADP exchange in such digitonin preparations has been studied in much more detail by Wadkins and Lehninger (80) and in mitochondria by Wadkins (77).

This exchange, measured either with P^{32}- or C^{14}-labeled ADP (or ATP) has a high rate relative to oxidative phosphorylation, is specific for the di- and triphosphates of adenosine, does not require phosphate, and is clearly distinct from other known reactions catalyzing such exchanges, as determined by enzymatic tests (80). Digitonin particles have only traces of adenylate kinase activity. Because the ATP-P_i^{32} exchange requires the presence of ADP, but the ATP-ADP exchange does not require P_i, the following formulation was postulated (52, 53) as representing the sequence of the terminal reactions of energy coupling:

(4) $$X \sim E + P_i \rightleftharpoons P \sim E + X$$

(5) $$P \sim E + ADP \rightleftharpoons ATP + E,$$

in which R ~ E represents a high-energy compound generated during electron transport and E, a phosphate-transferring enzyme. The formulation postulates a phospho-enzyme intermediate (P ~ E), occurrence of which is consistent with known isotopic data but which is as yet unproved. Reversibility of the two reactions would account for the two exchanges: reactions 4 + 5 for the ATP-P_i^{32} exchange and reaction 5 alone for the ATP-ADP exchange, and also for the fact that P_i is not required for the ATP-ADP exchange. The findings rule out the alternative sequence of ADP

and P_i uptake:

(6) $X \sim E + ADP \rightleftharpoons ADP \sim E + X$

(7) $ADP \sim E + P_i \rightleftharpoons ATP + E.$

Further study of the response of the ATP-ADP exchange to uncoupling agents and aging (53, 80) revealed an approach to separation of the enzyme catalyzing the reaction. In fresh digitonin fragments the exchange is quite sensitive to DNP, dicumarol, and gramicidin, as mentioned before. The exchange reaction is quite stable to aging at 0°, but the important finding was made that it loses its sensitivity to DNP on aging. This indicates that the ATP-ADP exchange is not itself sensitive to the uncoupling agents but may be in equilibrium (in fresh phosphorylating particles) with a second reaction in the coupling mechanism which is the true site of action of DNP. This explanation is supported also by the finding (fig. 3) that the rate of loss of DNP sensitivity of the ATP-ADP exchange is almost identical with the rate of inactivation of the ATP-P_i^{32} exchange, or the ability to couple phosphorylation to electron transport. Therefore the ATP-ADP exchange is stable, but the coupled reaction with which it is in equilibrium is the DNP-sensitive, labile reaction of the coupling mechanism.

The sensitivity of the ATP-ADP exchange to DNP is not simply a matter of stimulating hydrolysis of ATP and draining off ATP from the reaction medium, with an increase in ADP concentration. The DNP of course does stimulate ATP-ase activity but large inhibitions of the ATP-ADP exchange in the presence of DNP are observed before any significant change of the ATP:ADP ratio occurs. Furthermore, addition of Mg^{++}, which stimulates ATP-ase far more than DNP (23, 28) does not inhibit the ATP-ADP exchange. Other ATP-ADP exchange reactions, such as the adenylate kinase, are not inhibited by DNP in such digitonin particles. The inhibition by DNP is best explained by assuming it lowers the steady-state concentration of the intermediate $P \sim X$ by causing breakdown of $R \sim X$ or a preceding energy-rich intermediate.

The stability of the ATP-ADP exchange enzyme and its easy dissociation from other reactions of the coupling mechanism by aging or inhibitors led to its successful separation in soluble form by extraction of acetone-dried digitonin fragments with buffer, with little loss in activity (80). The rather stable soluble enzyme was shown to require Mg^{++} or Mn^{++} following dialysis, and was inhibited by sulfhydryl reagents. In soluble form the enzyme showed no ATP-P_i^{32} exchange, ATP-ase activity, and was completely insensitive to uncoupling agents, such as DNP. The enzyme was purified nearly 100-fold by iso-electric and ammonium sulfate fractionation, followed by chromatography on DEAE-cellulose columns (78). The purified enzyme appears to approach homogeneity by the usual tests. During purification it retains its insensitivity to uncoupling agents and its

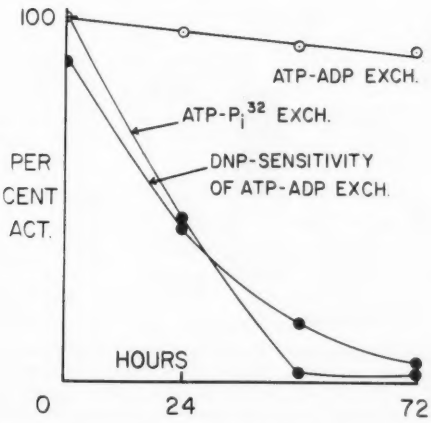


FIG. 3. Relationship between DNP-sensitivity of the ATP-ADP exchange reaction and the step in the ATP-P_i^{32} exchange and in oxidative phosphorylation which is labile to aging at 0°.

requirement for Mg^{++} or Mn^{++} and careful tests of the enzyme for occurrence of other phosphotransferase activities using appropriate substrates and cofactors are negative. In addition, the exchange is insensitive to avidin. Other physical and chemical properties of the purified enzyme will be described elsewhere (Wadkins, manuscript in preparation). It is also of significance that the soluble ATP-ADP exchange enzyme does not catalyze the incorporation or exchange of oxygen atoms between water and phosphate or water and ATP (12).

The possibility of the identity of the ATP-ADP exchange enzyme with the soluble ATP-ADP-P_i^{32} exchange enzyme isolated from mitochondria by Chiga and Plaut (18) must be considered. The latter enzyme, highly purified, catalyzes *both* ATP-ADP and ATP-P_i^{32} exchanges, whereas the Wadkins enzyme catalyzes only the nucleotide exchange. The Chiga and Plaut enzyme requires Mn^{++} and is only very slightly active with Mg^{++}; the Wadkins enzyme is about equally reactive with both ions. Finally, the Wadkins enzyme is far more active; at the highest purity (72-fold) it catalyzes the exchange of 15.0 μmoles of ADP per mg N. per min. whereas the Chiga and Plaut enzyme (250-fold purified) about 0.9 μmoles ADP per mg N. per min. The two groups have concluded the enzymes are probably not identical.

Recombination of soluble ATP-ADP exchange enzyme with the DNP-sensitive step. With the soluble, purified ATP-ADP exchange enzyme available, attempts were made to recombine this enzyme with preceding enzymes of the coupling sequence, as they occur in digitonin fragments. If the reaction pattern given by reactions (4) and (5) is in principle correct, then the exchange enzyme $P \sim E$ is capable of interacting not only with ADP but also with the molecule R. The rationale of 'recoupling' the soluble ATP-ADP exchange enzyme then is as follows. If digitonin fragments could be pretreated first to remove their complement of the ATP-

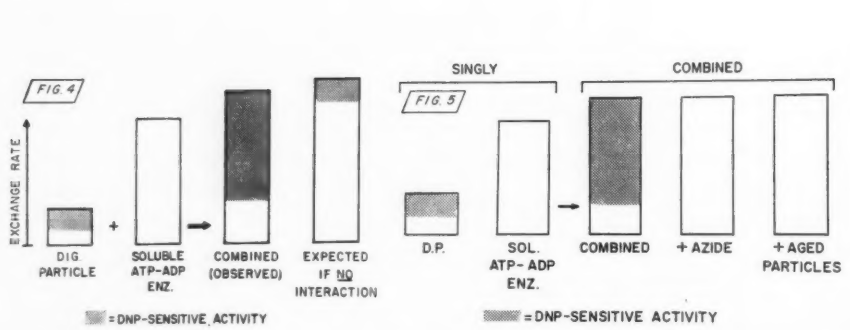


FIG. 4. 'Recombination' of soluble ATP-ADP exchange enzyme with digitonin fragments. Addition of fresh digitonin fragments to the DNP-insensitive, soluble enzyme confers DNP sensitivity on it. Had no interaction occurred between particles and soluble enzyme, the latter would remain DNP-insensitive The height of the bars indicates total ATP-ADP exchange activity; the shaded portion indicates fraction which is inhibited by 4×10^{-5} *M* DNP.

FIG. 5. Properties of 'recombination' reaction. Shaded portions of bars show extent of DNP-sensitivity before and after 'recombination,' in presence of azide-treated or aged digitonin fragments.

FIG. 6. 'Recombination' of purified versus crude ATP-ADP exchange enzymes.

ADP exchange enzyme, then it would appear possible to add back the soluble purified ATP-ADP exchange enzyme to reconstitute a complete system. One of several possible criteria for 'recombination' would be the restoration of DNP-sensitivity to the isolated ATP-ADP exchange enzyme, which in its soluble form is insensitive to DNP, the DNP-sensitive reaction to be contributed by the digitonin fragments. Such 'recombination' was found to be possible experimentally with either fresh or pretreated digitonin particles.

Data in figure 4 show that addition of a soluble but relatively impure sample of ATP-ADP exchange enzyme, which is DNP-insensitive, to a fresh digitonin particle preparation, in which the inherent ATP-ADP exchange was about 65% sensitive to DNP, caused the conferral of a large measure of DNP-sensitivity on the added soluble exchange enzyme, in full confirmation of the working hypothesis. Data in figure 5 show that the conferral of DNP-sensitivity on the soluble enzyme by the fragments did not occur when azide was present or when the digitonin particles were aged beforehand to the point of complete loss of activity in coupled phosphorylation, in agreement with the effects of azide and aging on the DNP-sensitivity of the ATP-ADP exchange in the unfractionated digitonin particles. These experiments thus indicate the feasibility of 'reattaching' the soluble ATP-ADP exchange enzyme to the relatively organized and complex digitonin fragments of the mitochondrial membranes in such a way as to reestablish an enzymatic connection with the DNP-sensitive reaction of the coupling mechanism. Presumably the particles contain 'empty' molecular sites which have lost the ATP-ADP exchange enzyme during preparation. Other experiments showed that by pretreating the digitonin particles with excess digitonin, virtually all the endogenous ATP-ADP exchange activity can be removed. Such particles also can restore DNP-sensitivity to added soluble ATP-ADP exchange enzyme.

On further examination of the 'recombination' reaction, using more purified specimens of the ATP-ADP exchange enzymes, it was found, surprisingly, that the highly purified enzyme did not 'recombine' with the digitonin particles, i.e. the digitonin particles did not confer DNP-sensitivity on purified ATP-ADP enzyme but did confer sensitivity on the exchange enzyme present in crude preparations or extracts (fig. 6). The most obvious explanation is that the crude preparation of ATP-ADP enzyme contains a factor or factors, in addition to the exchange enzyme, which is necessary to 'recombine' the ATP-ADP exchange enzyme, and which is lost on purification of the latter. Further experiments revealed the likelihood that this explanation was correct. Experiments in table 1 show that of the four ammonium sulfate fractions obtained from a soluble extract of acetone-dried mitochondria particles, fraction III contains most of the ATP-ADP exchange enzyme. However this partially purified enzyme does not 'recombine' with digitonin particles, (i.e. DNP-sensitivity was not conferred on the ATP-ADP exchange). Similarly, a 66-fold purified specimen of ATP-ADP enzyme did not recombine. However, when the sum of fractions I-IV is added to the digitonin particles, then DNP-sensitivity is conferred on the ATP-ADP exchange activity of the extract. The activity present in the crude extract necessary for recombining the ATP-ADP enzyme is designated as M-factor (or 'middle-factor') activity. Preliminary experiments indicate that this is present in fraction I and II; it is under further purification. Experiments described below furnish an independent assay for this material.

Restoration of DNP-sensitivity of the ATP-ADP exchange in digitonin fragments. A second property distinguishes M-factor preparations. It will be recalled that the ATP-ADP exchange as it occurs in fresh, unsupplemented digitonin fragments, is sensitive to DNP. However this sensitivity is not always complete, i.e. in some preparations no matter how high the DNP concentration, there is sometimes a significant residue of

TABLE 1. *Distribution of Enzyme Activities in Ammonium Sulfate Fractions of Extracts of Acetone-Dried Mitochondria*

Enzyme Activity	Fraction			
	I	II	III	IV
ADP* ⇄ ATP	o	o	++++	o
M-factor	++	++	o	o
C-Factor	++	++	o	o
Adenylate kinase	o	o	o	+++

uninhibited ATP-ADP exchange (80). While this exchange could be caused by adenylate kinase or other DNP-insensitive ATP-ADP exchange reactions, it seemed possible that it represented occurrence of the ATP-ADP exchange enzyme of oxidative phosphorylation in the particles which is 'dislocated' or disconnected from the DNP-sensitive reaction by lack of M-factor. Thus in fresh digitonin particles there may be empty sites normally occupied by the hypothetical M-factor, just as there appear to be empty sites normally occupied by the ATP-ADP exchange enzyme. It might be predicted that addition of soluble M-factor preparations to normal digitonin particles, in which DNP-sensitivity of the endogenous ATP-ADP exchange is incomplete, might increase the DNP-sensitivity. This was found to be the case (fig. 7); it is seen that addition of fractions I and II increase the DNP sensitivity of the endogenous ATP-ADP exchange quite significantly (81).

Thus two types of assay system measuring conferral of DNP-sensitivity on endogenous or on added ATP-ADP exchange enzyme, may be used to test M-factor. These properties of M-factor are reminiscent of the apparent role of polynucleotides in promoting the DNP-sensitivity of oxidative phosphorylation in extracts of *A. faecalis* studied by Pinchot (64). However polynucleotide preparations have no noteworthy effect on oxidative phosphorylation in digitonin fragments and crystalline ribonuclease added in large amounts has no uncoupling action.

We have postulated M-factor to be an intermediate enzyme in the energy-coupling mechanism, which may act between the ATP-ADP exchange reaction and the DNP-sensitive reaction. It could be identical with X, the entity first forming the energy-conserving linkage in respiration (81). However this is by no means a unique rationalization of the properties of M-factor. Actually it may only represent a type of molecular 'glue' necessary to refasten the ATP-ADP exchange enzyme to the membrane fragment in some way as to cause its reaction with the DNP-sensitive component, without reacting itself as an enzyme.

Reconstitution of oxidative phosphorylation. These successes in reconstitution of DNP-sensitivity on the ATP-ADP exchange reaction, using soluble and somewhat purified fractions, focused attention on the possibility that these fractions might also furnish the building stones for reconstitution of the overall process of oxidative phosphorylation, more specifically the restoration of the ability of membrane fragments to couple respiration and phosphorylation of ADP, as measured by an increase in P:O ratio in preparation in which ability to phosphorylate has been impaired by deletion of the terminal enzymes. Actually the requirement of soluble fractions in increasing P:O ratios of respiring fractions has already been observed by Pinchot in extracts of *A. faecalis* (63), and later by Brodie and Gray (11) in extracts of *M. phlei*. Recently Linnane and Titchener (54) have purified a soluble protein factor which raises the P:O ratio of pretreated beef heart particles. In these cases however there is little information available on the chemical and enzymatic reactions of the soluble fraction which might reveal the outlines of reaction pattern or mechanism.

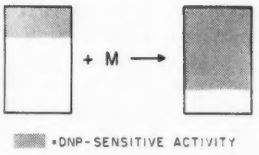


FIG. 7. Action of M-factor on DNP-sensitivity in fresh digitonin fragments. The soluble M-factor protein increases the DNP-sensitivity of the ATP-ADP exchange, but does not affect the total exchange rate.

On the other hand, the recent studies of Pullman, Penefsky and Racker (66) give more promise of revealing the mechanism of coupling. They have obtained a highly purified soluble protein factor from beef heart mitochondria which restores a limited amount of phosphorylation to nonphosphorylating beef heart particles (the increase in P:O ratio is reported to be from a baseline of 0.2 to a value of 0.6). This factor has been carefully analyzed for pertinent enzymatic activities and has been found to be devoid of activity in the ATP-ADP and ATP-P_i^{32} exchange reactions. On the other hand the factor does show DNP-stimulated ATP-ase activity which parallels the activity in restoring the P:O ratio during purification. These findings are rather important; they are quite different from the properties of the ATP-ADP exchange and the M-factor preparations we have studied. However, further comparison at this stage cannot be made until further data are available on the exchange reactions occurring in the 'recombined' system, i.e. the combination of soluble fraction plus respiring fragments.

The restoration of the P:O ratio of pretreated digitonin particles oxidizing β-hydroxybutyrate by soluble mitochondrial proteins has been extensively studied in our laboratory (Gregg, Wadkins, Lehninger, unpublished observations). By exposing such particles to excess digitonin and other reagents, the P:O ratio, normally 1.5–2.4, is greatly depressed. Such particles have also lost over 90% of their ATP-ADP exchange activity. When such particles are supplemented with crude extracts of acetone-dried mitochondria (containing fractions I–IV) the P:O ratio can be significantly restored, as shown by the data in table 2. The active factor is heat-labile. Furthermore the enzymatic identity of the restoring factor now seems to be within sight. Data in table 2 show that restoration of the P:O ratio in such pretreated digitonin fragments can also be

TABLE 2. *Restoration of Oxidative Phosphorylation by Soluble Factor*

System: Treated digitonin particles, β-hydroxybutyrate, P_i, ADP, serum albumin, Mg^{++} and additions shown.

System	−ΔP	−ΔO	P/O
1. —	100	175	0.4
+ Fraction I–IV	290	150	1.9
2. —	100	150	0.65
+ Fraction I–IV	200	125	1.60
+ Heated fraction	95	120	0.80
3. —	104	130	0.80
+ ATP-ADP enzyme	206	120	1.72

Data in mμmoles.

brought about by addition of a soluble, 66-fold purified ATP-ADP exchange enzyme. This finding provides suggestive evidence that the ATP-ADP exchange activity is part of the normal coupling machinery in oxidative phosphorylation. However, it is still quite possible that the 66-fold purified ATP-ADP exchange enzyme contains a factor in addition to or instead of the exchange enzyme which may be necessary to reconstitute the P:O ratio. Further purification will permit more decisive experiments. It is quite clear that this development, taken with the work of Pullman *et al.* (66) may soon bring important light on the mechanism of at least the terminal stages of the mechanism of energy coupling in oxidative phosphorylation.

Relationship of mitochondrial swelling and contraction to the mechanism of energy coupling. Recent progress in studying the factors involved in mitochondrial swelling and contraction indicates that these physical phenomena are reflections of the activity or state of the respiratory chain and the energy coupling mechanism and that they may provide important clues on the mechanism of coupling. Swelling of liver mitochondria induced by specific agents such as phosphate, Ca^{++}, thyroxine, glutathione, and U factor (see 42, 50, 59), requires that electron transport is taking place, since swelling does not occur anaerobically or when respiration is inhibited by cyanide, antimycin A, or amytal (17). Aged mitochondria no longer swell, because of depleted endogenous substrates, but can be induced to swell on addition of substrates. When the mitochondria are treated initially with dinitrophenol, the swelling response is also prevented (cf. 42 for recent review). These properties clearly indicate that respiration or respiration-generated intermediates, possibly in a specific oxidation-reduction state, are necessary for the change in physical state leading to water uptake and swelling. Evidence has been presented that swelling leads to increased permeability to sucrose (50).

The reversal of swelling, leading to contraction with concomitant water extrusion, also involves respiration, but less directly (cf. 42 for review). Earlier work by Davies *et al.* (65) and Beyer *et al.* (4) had shown that swollen mitochondria contract on institution of phosphorylating respiration, an effect studied more closely by Packer (62). However, contraction of mitochondria may occur in the presence of cyanide or dinitrophenol, provided ATP is added (46, 47), indicating that the role of respiration in mitochondrial contraction is that of generating ATP or an immediate precursor of ATP with which it may be in equilibrium. The contraction is specific for ATP among the nucleoside 5′ triphosphates tested, although ADP sometimes gives a much slower contraction, presumably caused by action of adenylate kinase (62). However it is possible that the active nucleotide is intramitochondrial and that addition of external nucleotides conditions the level of internal nucleotides. The ATP-induced contraction is inhibited by azide and also by sucrose; both these compounds are inhibitors of an intermediate step in the coupling of phosphorylation and respiration (53). This fact indicates that contraction involves a step in the coupling mechanism between the point of DNP action and the final formation of ATP (42). The inhibition by sucrose is interesting; it is apparently not due to impurities. Inhibition of contraction is also given by 0.3 M concentrations of a variety of polyols, such as glucose, fructose, raffinose, inositol, mannitol, sorbitol, xylose, and dextran, but not by simpler alcohols such as glycerol, ethylene glycol, ethanol, etc. (48). Polyols which inhibit contraction also inhibit the DNP-stimulated ATP-ase and the ATP-P_i^{32} exchange in digitonin fragments (48), indicating that there is a common denominator shared between contraction and coupling. The fact that the osmotically insensitive digitonin particles are inhibited by polyols would suggest a chemical inhibition rather than an osmotic effect.

One of the most significant developments to come from studies of swelling and contraction, which may lead to chemical identification of part of the contraction mechanism and possibly also identification of an intermediate step in oxidative phosphorylating, is the separation of a soluble mitochondrial protein (C-factor, for 'Contraction-factor') which is necessary for contraction by ATP (49). This protein is detached or caused to 'leak' into the medium from rat liver mitochondria swollen by reduced glutathione. Mitochondria swollen by GSH do not contract on addition of ATP + Mg^{++} + serum albumin, presumably because of the loss of C-factor to the medium. When the factor is recovered from the medium and added back to GSH-swollen mitochondria in suitable concentrations, then ATP induces contraction. The C-factor is heat-labile, does not dialyze, and may be purified by acetone or $(NH_4)_2SO_4$ fractionation or by chromatography on DEAE-cellulose columns. Progress of purification is assayed by the ability to promote contraction of GSH-swollen mitochondria. As figure 8 shows, additions of increasing amounts of C-factor result in increasing extent of contraction of the mitochondria.

It naturally became of interest to learn if C-factor, a soluble mitochondrial protein, is identical with any of the known soluble or solubilized mitochondrial proteins

FIG. 8

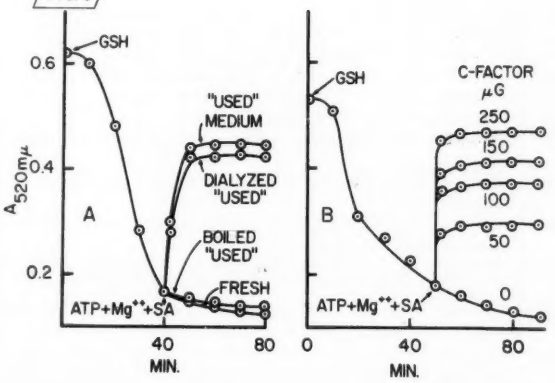


FIG. 8. Effect of C-factor protein on contraction of GSH-swollen mitochondria. At left is shown the failure of GSH-swollen mitochondria to contract in the presence of ATP and the effect of adding some of the medium in which mitochondria in high concentration had been allowed to swell (used medium). The C-factor in the medium is not dialyzable and is heat-labile. At right is shown the effect of increasing C-factor concentration.

FIG. 9

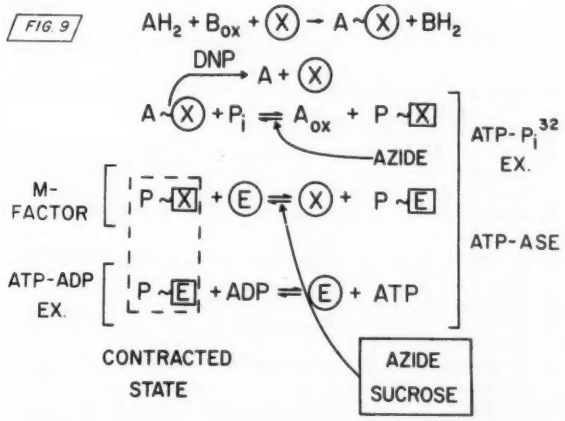


FIG. 9. Possible relationships between the coupling reactions of oxidative phosphorylation and the physical state of the mitochondrial membranes. In this representation of the partial reactions the phosphoenzymes P ~ [X] and P ~ [E] are in the 'contracted' configuration, indicated by squares. The 'relaxed' configuration is given by the symbols (X) and (E) for the dephosphorylated enzyme. Alternatively, an additional membrane protein Pr, not itself in the coupling sequence, may become phosphorylated and change from 'relaxed' (Pr) to 'contracted' P ~ [Pr] configuration.

which are concerned in energy coupling, such as the M-factor and the ATP-ADP exchange enzyme described above. It was found that the 66-fold purified ATP-ADP exchange enzyme does not possess C-factor activity, nor does adenylate kinase (table 3), serum albumin, hexokinase, and a variety of other proteins likewise are inactive. However, it was found that C-factor activity is present in those fractions of mitochondrial protein, extracted by phosphate from acetone-dried mitochondria which also contain the M-factor activity, namely fractions I and II. This finding thus suggests the possibility that C-factor, required in the ATP-linked contraction of mitochondria, may be identical with M-factor, which is in turn necessary to confer DNP-sensitivity on the ATP-ADP exchange. Further purifications of C-factor and M-factor should therefore reveal whether these entities are identical. If this should be the case, it will provide an important opening to the biochemistry of the contractile process in mitochondria.

We have already postulated (42, 46, 47, 50) that one

TABLE 3. *Distribution of C-Factor Activity**

	Units/mg N
Serum albumin (bovine)	0
Serum γ-globulin	0
Fraction I + II	20
Fraction III	1
Fraction IV (adenylate kinase)	2
ATP-ADP exchange enzyme	2
DEAE-cellulose fraction	500
Yeast hexokinase	0

* D. Neubert and A. L. Lehninger, full account to be published.

or more of the energy-coupling enzymes in the respiratory chain may be 'mechanoenzymes' which can change physical configuration as a function of their state (fig. 9). Such 'mechanoenzymes' would be analogous to actomyosin and as a matter of fact there appear to be interesting parallels in the ATP-ase activity of phosphorylating fragments of mitochondria and of actomyosin and its components. A change of conformation could be produced by binding of ATP or by the formation of a phosphoenzyme, or by changes in charge distribution. Such changes may be detectable by appropriate physical methods, such as light-scattering, viscosity, and electrokinetic approaches, which we are now applying to the purified coupling enzymes, such as the ATP-ADP enzyme.

In conclusion, the intimate mechanism of oxidative phosphorylation, particularly the chemistry of the primary formation of a carrier-linked 'high-energy' bond which conserves energy of electron transport, is still a mystery, but with the recent findings that soluble, specific proteins from mitochondria are capable of affording reconstitution of both coupled phosphorylation and the so-called 'partial reactions' of oxidative phosphorylation some optimism is justified that the outer ramparts of this very formidable problem may finally be crumbling.

In the original work from this laboratory reported here, Drs. Charles L. Wadkins, Charles Gregg, Diether Neubert, Shinji Ishikawa, Gerald Gotterer, Thomas Thompson, and Lech Wojtczak were principal colleagues. The National Institutes of Health, the National Science Foundation, and the Nutrition Foundation, Inc. and The Whitehall Foundation supported our studies generously.

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Folic acid coenzymes

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THE HISTORY OF THE DISCOVERY and proof of structure of the vitamin folic acid is summarized in the review by Stokstad (29). The discovery of the 'citrovorum factor' by Sauberlich and Baumann (27) and its subsequent identification as 5-formyl-THF[1] by May *et al.* (16) and by Roth *et al.* (26) suggested the possible role of the vitamin as a one-carbon carrier in the form of its tetrahydro-derivative. The coenzyme function of tetrahydrofolic acid and its derivatives has been discussed in two recent reviews (12, 20). The present discussion will present a brief summary of the enzymic reactions which involve folic acid coenzymes and will treat several more limited aspects of the role of the folic acid coenzymes which have appeared since the publication of these reviews.

ROLE OF FOLIC ACID COENZYMES

The metabolic systems known to be dependent on folic acid coenzymes are listed in table 1. Each system involves a one-carbon moiety, although the oxidation level of this moiety varies. Many of the enzymes involved in these folic acid-dependent systems have been purified and the chemical nature of the one-carbon derivatives of the folic acid coenzymes involved in the specific enzymic reactions has been determined.

The details of the reactions involving the folic acid coenzymes with the CH_3 group are still not known in detail, although reports related to this function have recently appeared (5, 9, 11, 15). Our knowledge concerning the next three types of coenzyme derivatives is much more extensive.

The reactions of the hydroxymethyl derivative or formaldehyde derivative of THF are summarized in figure 1. An adduct of formaldehyde and THF is formed either nonenzymically (13) or enzymically (18). The structure of the compound formed is shown here as the 5,10-methylene-THF and is based largely on the work of Blakley (4), Kisliuk (13) and Osborn *et al.* (18). The 5,10-methylene-THF may transfer its hydroxymethyl group to glycine to form serine in the presence of the enzyme serine hydroxymethylase and the cofactor pyridoxal phosphate, as originally shown by Kisliuk and Sakami (14) and Blakley (4). The reaction is reversible and obviously serine can also act as a source of 5,10-methylene-THF in the presence of THF and the enzyme. The oxidation of the methylene group of 5,10-methylene-THF to yield 5,10-methenyl-THF is catalyzed by a TPN linked dehydrogenase first demonstrated by Kisliuk and Sakami (14) and Blakley (4). In this reaction, the one carbon moiety is oxidized from the formaldehyde oxidation level to that of formic acid. More recently, Scrimgeour and Huennekens (28) have described a DPN-specific 5,10-methylene-THF dehydrogenase.

The enzymic reactions involving the one carbon unit at the formate oxidation level are summarized in figure 2. The 5,10-methenyl-THF is converted to 10-formyl-THF at neutral pH in a non-enzymic reaction (16), or by the enzyme cyclohydrolase (22). An active formyl-THF may also be formed in a reaction between formic acid, THF, and ATP. The products of the reaction have been identified as 10-formyl-THF, ADP, and orthophosphate, and the enzyme responsible for the reaction is called THF-formylase (8).

It was not possible to determine which chemical form of formyl-THF participates in the enzymic transformylation reactions involved in purine biosynthesis as long as the enzymes were contaminated with cyclohydrolase. It was evident, however, from the enzymic studies of G. R. Greenberg that 5-formyl-THF is not the active transformylating factor (6). Buchanan and his coworkers have recently purified two transformylase enzymes involved in purine biosynthesis sufficiently so that they are free of cyclohydrolase. These investigators found that 10-formyl-THF acts as the formyl donor with amino imidazole carboxamide ribonucleotide, while 5,10-methenyl-THF acts as the formyl donor with glycinamide ribonucleotide to form the N-formyl glycinamide ribonucleotide (10). Thus, either 10-formyl- or 5,10-methenyl-THF may act as the active formyl donor with the appropriate enzyme system.

The reactions involved in the formation of the formimino derivatives of THF are shown in figure 3. Formiminoglycine is formed in the degradation of purines by certain *Clostridia* (24). In the presence of the enzyme formiminoglycine formimino transferase, the formimino group is transferred to THF to form 5-formimino-THF. This product is then converted to 5,10-methenyl-THF and ammonia by the enzyme cyclodeaminase. The deamination and ring formation occur simultaneously since the deaminated product —5-formyl-THF—is not an

[1] The abbreviation THF has been used for tetrahydrofolic acid. The various one-carbon derivatives of this compound are indicated by the unabbreviated prefix for which the chemical formula is given in table 1. The numbers preceeding the prefix refer to the position of substitution on the pteridine ring.

TABLE 1. *Metabolic Systems Dependent on Folic Acid Coenzymes*

System	Chemical Moiety Implicated	
Methionine biosynthesis Thymine biosynthesis	$-CH_3$	methyl-
Serine biosynthesis 5-Hydroxymethyl-Cytosine biosynthesis	$-CH_2OH$	hydroxymethyl- methylene- (formaldehyde)
Purine biosynthesis Valine biosynthesis	$-CHO$	formyl- methenyl- (formic acid)
Purine fermentation Histidine degradation	$-CH=NH$	formimino-

intermediate in this reaction (23). The analogous formimino transfer reaction involving formiminoglutamic acid has been demonstrated and purified from mammalian tissues by Tabor and associates (30, 31).

THE FOLIC ACID COENZYME

THF has been used extensively as the folic acid coenzyme in the enzymic reactions described in the previous section. THF has been used in these studies largely as a matter of convenience, since it is readily prepared by the catalytic reduction of folic acid, first described by O'Dell *et al.* (17). However, the evidence available using microbiological assay techniques has clearly shown that neither THF nor folic acid occurs as such in natural materials, and suggests that THF cannot be the naturally occurring form of the folic acid coenzyme. The results of these earlier investigations indicate that all the vitamin occurs as a reduced folic acid derivative, and that it occurs as a polyglutamic acid conjugate. The fact that all the folic acid derivatives which have been isolated from natural sources have been oxidized compounds rather than tetrahydro derivatives, with the single exception of 5-formyl-THF, results in large part from the fact that THF and its derivatives which are not substituted in the 5-position are extremely oxygen labile (2), although solutions of THF can be stabilized by the addition of reducing agents such as mercaptoethanol or ascorbic acid. It, therefore, became desirable to determine the chemical nature of the naturally occurring folic acid coenzyme and to isolate it, if possible, in order to compare it to THF with respect to enzymic activity.

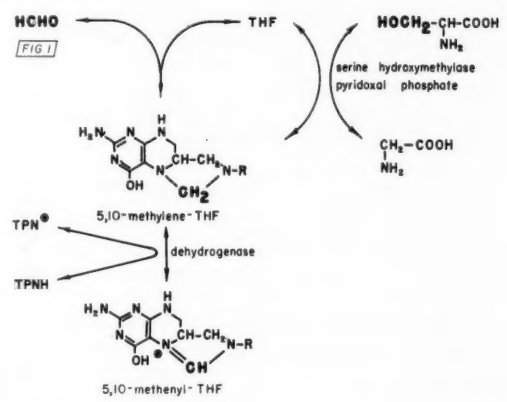
The purine fermenting *Clostridia* were used as a source of the coenzyme since these organisms contain an unusually large amount of folic acid—about 200 times more than mammalian liver, the common starting material for the isolation of folic acid and its derivatives. It was found by enzymic assay that the predominant part of the folic acid coenzyme occurs as an unsubstituted compound, and that only negligible amounts occur as the formyl-, methenyl-, or formimino-derivatives. Thus, in order to isolate the coenzyme, it is necessary to prevent destruction of the cofactor through air oxidation. Extracts were therefore prepared by boiling the cells in 1 *M* mercaptoethanol and 25% ethanol to prevent oxidative and enzymic destruction of the cofactor. The cofactor was then converted to the oxygen stable formimino derivative using the purified formiminoglycine formimino transferase, and the compound was then chromatographed on diethylaminoethyl cellulose (21). The results of the chromatography are shown in figure 4. The column was developed with a gradient of phosphate buffer at pH 6, as indicated by the diagonal line. The formimino derivative of THF is eluted with 0.05 *M* phosphate and is easily separated from the formimino derivative of a synthetic sample of tetrahydropteroyl-triglutamic acid, which is eluted with 0.20 *M* phosphate buffer. Although the cell extract yielded many components with absorption at 280 mμ, only one of these reacted in the enzyme test, and this component is eluted with 0.20 *M* phosphate. It is similar in this respect to the formimino derivative of tetrahydropteroyl-triglutamic acid. This component represents 80% of the activity placed on the column and from 75 to 80% of the material originally present in the cell extracts. No activity at all could be detected in the region where the formimino derivatives of THF, itself, would be expected to appear.

Encouraged by these results, we attempted to chromatograph the cell extract on the diethylaminoethyl-cellulose column without prior conversion to the formimino derivative. This could be done without destroying THF by incorporating 0.2 *M* mercaptoethanol into all the solutions used. Under these conditions, THF was recovered in 80 to 83% yield, as determined by enzymic assay using THF-formylase. THF is eluted with 0.03 *M* phosphate buffer, and the tetrahydropteroyl-triglutamic acid is eluted with 0.15 *M* phosphate. Chromatography of the cell extract is shown in figure 5. A single component with enzymic activity was eluted with 0.15 *M* phosphate. No activity at all could be detected in the region where THF should be eluted.

Since the higher glutamic acid conjugates of THF were not available to us, we were unable to determine whether these compounds could be distinguished from the triglutamic acid derivatives by this chromatographic method. The glutamic acid content of the tubes was therefore determined after acid hydrolysis, using glutamic acid dehydrogenase. The ratio of glutamic acid to pteridine was very close to 3 in all portions of the peak, showing that the compound was homogeneous and contained three glutamic acid residues per pteridine moiety.

The structure of the folic acid coenzyme which occurs in these *Clostridia* based on the results presented is shown in figure 6. The glutamic acid residues are shown in γ linkages by analogy to the structures of the polyglutamyl derivatives of folic acid which have been previously isolated and characterized by the Lederle group, and by Pfiffner *et al.* (19).

The activity of the natural cofactor and of THF were compared in three enzymic reactions as shown in table 2. The Michaelis constant and the maximum reaction



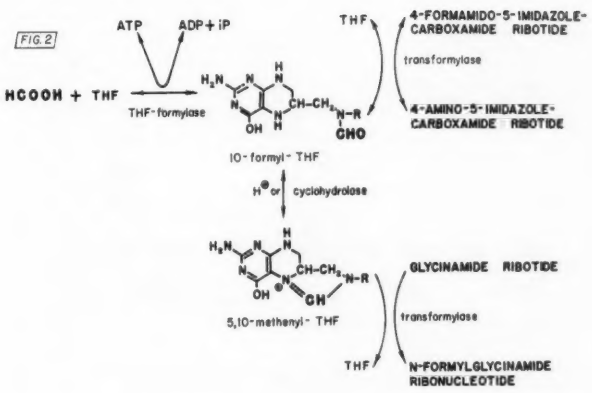
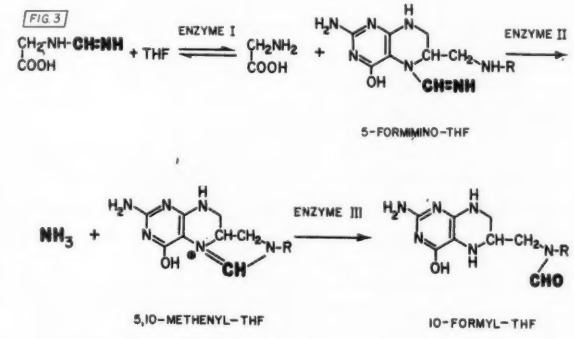


FIG. 1. Enzymic reactions of 5,10-methylene-THF.
FIG. 2. Enzymic reactions of 5,10-methenyl-THF.
FIG. 3. Enzymic reactions of 5-formimino-THF.



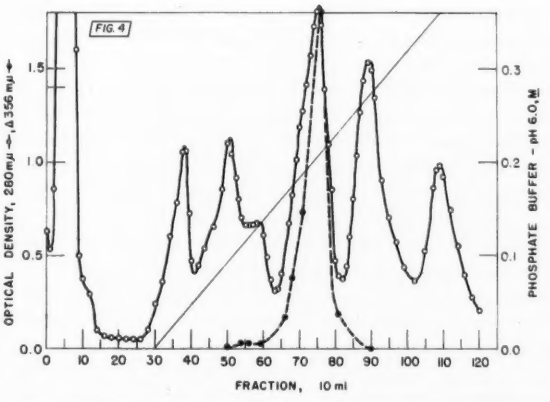


FIG. 4. The chromatography of the formimino derivatives of extracts of *Clostridium acidi-urici* on diethylaminoethyl cellulose. The open circles are the absorbance at 280 mμ, and the closed circles are the increase in absorption at 350 mμ after treatment of an aliquot of the fraction with cyclodeaminase.

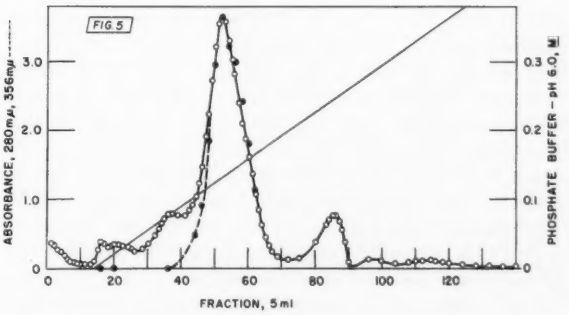


FIG. 5. The chromatography of an extract of *Clostridium acidi-urici* on diethylaminoethyl cellulose. The open circles are the absorbance at 280 mμ, and the closed circles are the increase in absorption at 350 mμ under conditions used for the enzymic assay of THF using THF-formylase.

velocities were determined. The activity of the synthetic THF and the natural cofactor were very similar for the formiminoglycine formimino transferase, and the formimino derivates of the synthetic coenzyme and the natural coenzyme had essentially identical behavior for the cyclodeaminase. The reaction velocities observed with the two substances were also identical with THF-formylase; however, the affinity constant of the natural cofactor was 10 times greater than that of synthetic THF for the purified enzyme, THF-formylase. The requirement for polyglutamyl derivatives of THF has been reported for the serine hydroxymethylase of a *Clostridium* by Wright (33) and for the synthesis of methionine in *E. coli* extracts by Guest and Jones (9). In view of these

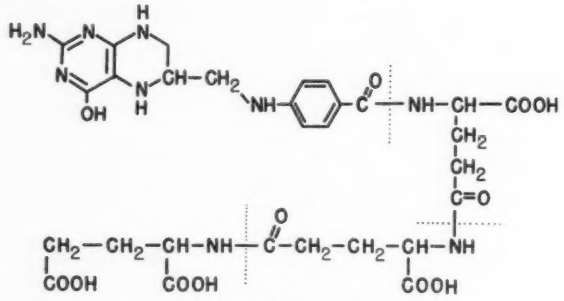


FIG. 6. Tetrahydropteroyl-triglutamic acid.

TABLE 2. *Properties of Synthetic and Natural Folic Acid Cofactors in Enzymic Reactions*

Enzyme	K_m M × 10^5		V_{max} Moles/l/ Min. × 10^6	
	l-THF	Natural cofactor	*l*-THF	Natural cofactor
Formimino transferase	2.4	1.4	0.84	1.18
Formimino-THF cyclodeaminase	1.1	0.9	18.5	12.5
THF-formylase	29	2.5	7.0	7.0

observations, chemically prepared THF should be regarded as a convenient synthetic analogue of the folic acid coenzyme, tetrahydropteroyl-triglutamic acid.

THF-FORMYLASE

The enzyme tetrahydrofolic acid formylase was first described by Greenberg and Goldthwaite in pigeon liver powders and has been purified about 30- to 40-fold from that source (7). The enzyme has also been purified near 1000-fold from *Micrococcus aerogenes* by Whitely, Osborn and Huennekens (32) and has been obtained in the crystalline form after only a 10-fold purification from extracts of the purine-fermenting *Clostridium*, in work done in collaboration with Mr. William E. Pricer (25). The reaction catalyzed by the enzyme is shown in figure 2 and this enzyme is usually regarded as the one responsible for the activation of formic acid. The product of the reaction, 10-formyl-THF, is easily determined after its conversion to 5,10-methenyl-THF with acid, by the absorption at 350 mμ. The crystalline enzyme was obtained using conventional purification techniques in about 50% yield. The over-all purification based on the dry weight of cells is about 32-fold. Assuming that the crystalline enzyme is pure, this would indicate that 3% of the dry weight of the cell is THF-formylase. This exceptionally high concentration of the enzyme in these organisms is probably related to the fact that this reaction, operating in the reverse direction from that indicated in figure 2, is the main energy yielding step in the fermentation of purines. The crystalline enzyme was shown to be homogeneous by sedimentation, with a sedimentation constant of 9.3, and the molecular weight calculated from this value and the diffusion constant is about 230,000.[2]

TABLE 3. *Equilibrium of THF-Formylase Reaction*

ATP + HCOOH + THF ⇌ 10-formyl-THF + ADP + iP

μmoles at Equilibrium						K_e	$-\Delta F$ Calories
l-THF	ATP	HCOOH	ADP	10-formyl-THF	iP		
1.6	0.2	5.7	3.2	3.3	3.3	20	1800
0.9	0.8	2.3	4.0	4.0	4.0	42	2200
0.2	1.4	2.6	3.5	3.5	3.5	61	2450

Av. 2150

TABLE 4. *Proposed Reaction Mechanisms for THF-Formylase*

ATP + THF + HCOOH ⇌ 10-formyl-THF + ADP + iP

I. THF "Activation":

ATP + THF ⇌ phosphoryl-THF + ADP
phosphoryl-THF + HCOOH ⇌ 10-formyl-THF + iP

II. Formate "Activation":

ATP + HCOOH ⇌ formyl-phosphate + ADP
formyl-phosphate + THF ⇌ 10-formyl-THF + iP

III. Acyl Adenylate Formation:

ATP + HCOOH ⇌ formyl-P-adenylate + iP
formyl-P-adenylate + THF ⇌ 10-formyl-THF + ADP

IV. Enigmatic ATP Reactions:

1. Glutamine synthetase
2. Succinyl thiokinase
3. Glutathione synthetase
4. Various enzymic reactions in purine biosynthesis.

Earlier in the discussion the suggestion was made that the reaction catalyzed by this enzyme is responsible for the formation of ATP in the purine fermentation. The results of studies of the equilibrium of this reaction, shown in table 3, support this view. In the three experiments shown, the reaction was allowed to come to equilibrium and the 6 components were determined. The equilibrium constant observed for the reaction is shown, and the free energy change calculated from this data gives an average value of about 2,000 calories. This indicates that the reaction is fairly readily reversible, and could, in fact, explain the formation of ATP by the organism.

The remainder of this discussion will be concerned with a consideration of the possible mechanism of the reaction catalyzed by the crystalline *Clostridium* enzyme. The reaction mechanisms first considered were those involving the formation of free intermediates, since these mechanisms suggested the most apparent experimental approaches to the problem. The reactions considered are

[2] The authors wish to thank Dr. H. Edelhoch of the National Institute of Arthritis and Metabolic Diseases, Bethesda, and Dr. H. K. Schachman of the Department of Biochemistry, University of California, Berkeley, for their generous advice and aid in these studies.

shown in table 4. They involve initial activation of either THF or HCOOH by ATP, to form either phosphoryl-THF, formyl-phosphate, or, by analogy to the acyl adenylates discovered by Berg (3), a formyl-phosphoryl-adenylate. In addition to these mechanisms, there are a large number of ATP reactions which have been studied extensively, which also involve ATP and two additional substrates, which do not fit very readily into any of the above mechanisms since the formation of reaction intermediates has not been demonstrated. These reactions have been classed together and called 'enigmatic' ATP reactions, although it remains to be determined whether they all proceed by a common mechanism.

One of the first experiments done was to determine the factors essential for the exchange of orthophosphate labeled with P^{32} and ATP. The results of our experiments are shown in table 5. In this experiment, ATP was isolated after incubation with THF, HCOOH, and P^{32}-orthophosphate and was shown to have 80% of the amount of P^{32} expected if the exchange were complete. The omission of any of the substrates decreased the exchange to a negligible level. The small amount of exchange noted on the omission of HCOOH is probably explained by contamination of the system with small amounts of formate, as shown by the formation of small amounts of 10-formyl-THF in this case. These results demonstrate that all 3 components are essential for the exchange of orthophosphate into ATP and therefore eliminate Mechanism III from further consideration.

Mechanisms I and II may be distinguished by determining the factors essential for the formation of ADP from ATP. If the reaction proceeds by an initial activation of THF, Mechanism I predicts a requirement for THF for the formation of ADP, whereas Mechanism II predicts a requirement for HCOOH. The formation of ADP can be detected spectrophotometrically by using phosphoenolpyruvic acid kinase and phosphoenolpyruvate, together with lactic acid dehydrogenase and DPNH; ADP formation is detected in the system from the resulting oxidation of DPNH. This assay system was used by Whitely, Osborn and Huennekens (32) to demonstrate ADP formation when ATP is incubated with THF in the presence of the THF-formylase purified from

TABLE 5. *Exchange of P^{32}-Orthophosphate into ATP by THF-Formylase*

ATP + HCOOH + *l*-THF $\rightleftharpoons$ *l*-10-formyl-THF + ADP + iP*

Components	10-Formyl-THF Formed μmoles	Radioactivity of ATP cpm/μmole
Complete	2.3	44,000
omit Mg^{++}	2.3	5,500
omit THF	0.00	0
omit HCOOH	0.03	1,200
omit Enzyme	0.00	0

* The complete system contained 5 μmoles each of ATP and sodium formate, 4.8 μmoles of *dl*-THF and 6 μmoles of potassium phosphate containing 124,000 cpm/μmole, in a volume of 1 ml. All tubes were incubated for 3.5 hr. at 37 °C.

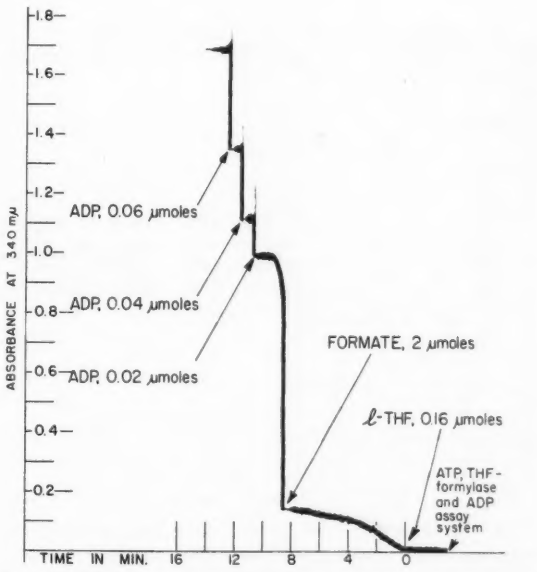


FIG. 7. ADP formation by THF-formylase. Cuvettes contained in 1 ml.: triethanolamine buffer at pH 8.0, 100 μmoles; $MgCl_2$, 10 μmoles; phosphoenolpyruvate, 1.5 μmoles; DPNH, 0.5 μmoles; ATP, 2.5 μmoles; 2-mercaptoethanol, 100 μmoles; phosphoenolpyruvate kinase, 70 μg.; lactic dehydrogenase, 80 μg.; and THF-formylase, 15 μg. (500 U). The reference cell contained everything except ATP. The reaction and reference cells were placed in a Cary Recording Spectrophotometer, Model 14B and the absorbance at 340 mμ was recorded. The additions indicated in the chart were made to the cuvette held in the compartment normally used for the reference cell, and thus DPNH disappearance is actually shown as an increase in absorbance.

Micrococcus aerogenes and led them to postulate the reaction mechanism for the THF-formylase reaction indicated by Mechanism I of table 4. The results obtained with the crystalline *Clostridium* enzyme are shown in figure 7. The change in absorbance at 340 mμ is plotted as a function of time, and the experiment was arranged so that the disappearance of DPNH or the formation of ADP is recorded as an increase in absorbance. The addition of ADP to the system results in the immediate increase in absorption at 340 mμ, as shown on the left hand part of the figure. When ATP is incubated with the ADP assay system and THF-formylase, no ADP formation is detectable. The addition of formate, not shown here but done in separate experiments, did not lead to the formation of any ADP. The addition of THF results only in the formation of a small amount of apparent ADP, but the reaction stops shortly. However, the addition of HCOOH, which gives the complete system, results in the immediate formation of ADP. This experiment, therefore, fails to support either reaction Mechanisms I or II, since it shows that ADP is only formed in the presence of the three reaction components.

A more critical evaluation of the THF activation mechanisms was made by determining the factors required for the exchange of ADP^{32} into ATP. The β-labeled ADP^{32} used was prepared by the photosyn-

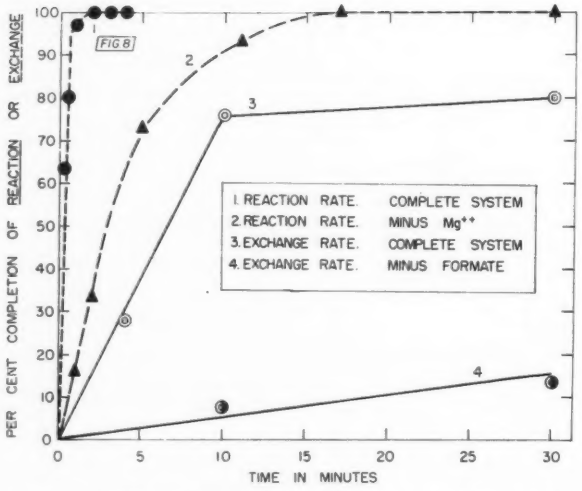


FIG 8
PER CENT COMPLETION OF REACTION OR EXCHANGE
TIME IN MINUTES
1. REACTION RATE. COMPLETE SYSTEM
2. REACTION RATE. MINUS Mg++
3. EXCHANGE RATE. COMPLETE SYSTEM
4. EXCHANGE RATE. MINUS FORMATE

FIG. 8. THF-formylase reaction rates and β-ADP^{32}—ATP exchange rates catalyzed by THF-formylase. The complete reaction mixture was incubated at 37° in 1 ml and contained: triethanolamine buffer at pH 8.0; 100 μmoles; 2-mercaptoethanol, 200 μmoles; sodium ascorbate, 20 μmoles; $MgCl_2$, 10 μmoles; sodium formate, 5 μmoles; *dl*-THF, 6.4 μmoles; ADP^{32}, 143,000 cpm/μmole, 5 μmoles; ATP, 5 μmoles; THF-formylase, 0.15 mg (5000 U) which had been dialyzed 18 hours against 0.05 *M* potassium maleate buffer at pH 7.0, 0.1 *M* with respect to 2-mercaptoethanol. After incubation the reaction was stopped by the addition of 2 ml of 0.36 *N* HCl. The amount of 10-formyl-THF formed was determined by measuring the change in absorbance at 350 mμ upon addition of the acid. The nucleotides were adsorbed on acid-washed Darco G, were then eluted with ethanol-ammonia. The nucleotides and pteridine degradation products were chromatographed on Dowex-1-Cl. The adenine nucleotides were collected and counted in a nuclear gas flow counter.

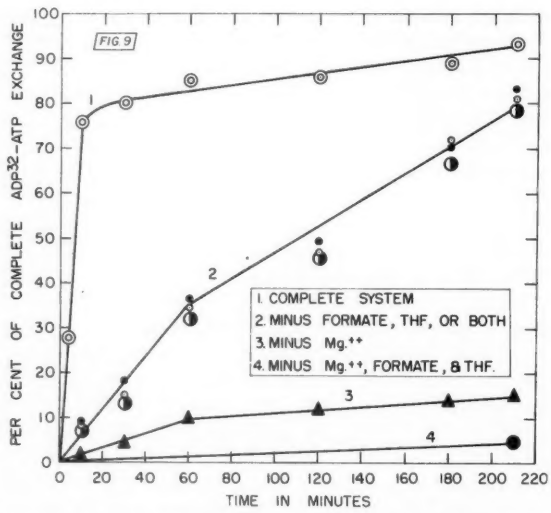


FIG 9
PER CENT OF COMPLETE ADP32-ATP EXCHANGE
TIME IN MINUTES
1. COMPLETE SYSTEM
2. MINUS FORMATE, THF, OR BOTH
3. MINUS Mg.++
4. MINUS Mg.++, FORMATE, & THF.

FIG. 9. β-ADP^{32}—ATP exchange rates catalyzed by THF formylase. The conditions used were identical to those described in figure 8.

thetic phosphorylation of AMP using spinach chloroplasts as described by Allen, Whatley and Arnon (1) and was shown to be labeled exclusively in the β- or terminal phosphate. The exchange experiment was done by incubating ATP, β-ADP^{32} and the enzyme with the various components of the system. Reaction Mechanism I predicts that the exchange should be dependent on the addition of THF.

The results of the experiment are shown in figure 8. The exchange rates observed were much slower than the over-all rate of the reaction. A small but significant exchange was observed between β-ADP^{32} and ATP when incubated with THF and enzyme in the absence of formate.

The ADP-ATP exchange rate is shown in figure 9 on a different scale under a variety of experimental conditions. The exchange rate observed between β-ADP^{32} and ATP was identical under conditions when formate, THF, or both THF and HCOOH were omitted. The results show that THF-formylase catalyzes an ATP-ADP exchange which is independent of the other components of the formylase reaction—and is, in fact, not affected by the other components. These results again fail to support either the THF-activation or the HCOOH-activation mechanisms, shown by Mechanisms I and II, and we may, therefore, classify the THF-formylase with the other enigmatic ATP reactions.

The ATP-ADP exchange reaction catalyzed by the crystalline enzyme could be caused by a number of contaminating enzymes. The enzyme was, therefore, examined for the presence of some of these activities under conditions similar to those used to study the exchange reaction. The enzyme was shown to be free of both adenosine triphosphatase and nucleoside diphosphokinase activity.

The absence of adenylate kinase was demonstrated by analyzing the ATP isolated from the reaction mixture.

TABLE 6. *Analysis of ATP Formed from β-ADP^{32} by THF-Formylase*

$$2A—R—P—P^* \rightleftharpoons A—R—P—P^*—P^* + A—R—P$$
$$\text{glucose} + \text{hexokinase}$$
$$A—R—P—P^* + \text{glucose-6-}P^*$$

Compound Analyzed	Radioactivity cpm/μmole	% of Labeling
ATP formed with THF-formylase	30,000	100
ADP formed after hexokinase	27,000	90
glucose-6-P formed after hexokinase	2,460	8

TABLE 7. *O^{18} Studies with THF-Formylase*

$HCO_2^{18}Na + ATP + THF \rightleftharpoons$ 10-formyl-THF + ADP + iP

Material Analyzed	Atom % Excess O^{18}	% of HCOONa
HCOONa	2.285	
KH_2PO_4	0.581	25.4
ADP	0	
ATP	0	

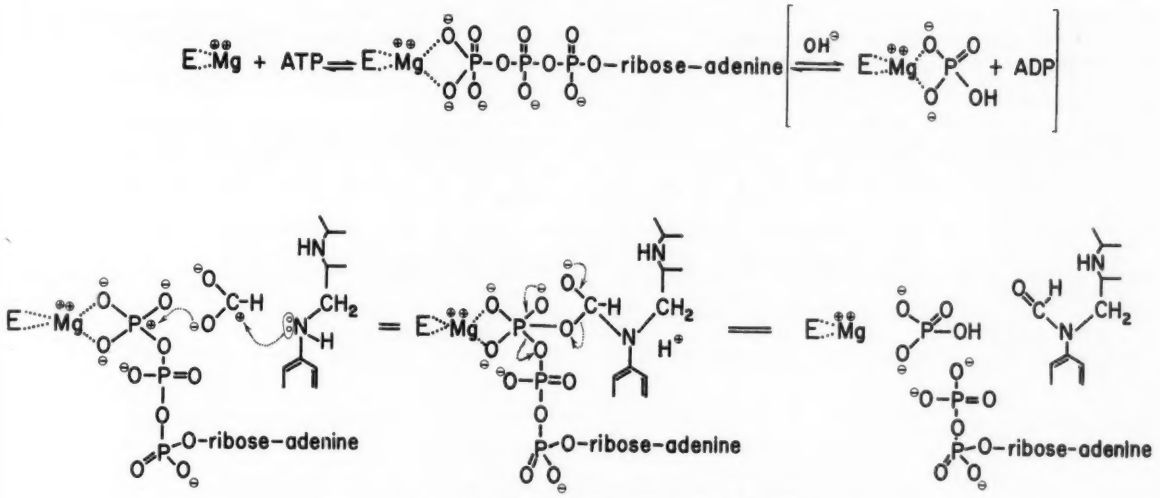


FIG. 10. Proposed reaction mechanism for THF-formylase.

As shown in table 6, adenylate kinase would be expected to act on β-ADP^{32} to form ATP labeled equally in the two terminal phosphates. The ATP isolated from the THF-formylase reaction was, therefore, degraded with glucose and hexokinase to yield glucose-6-P, representing the γ-phosphate, and ADP, which contained the β-phosphate. The glucose-6-P contained only 8% of the radioactivity of the ATP instead of 50% expected from the action of adenylate kinase.

Since the formylase is essentially free of ATP-ase, nucleoside diphosphate kinase, and adenylate kinase, we conclude that the exchange observed is catalyzed by THF-formylase.

Additional evidence concerning the reaction mechanism of THF-formylase was obtained by using formate labeled with O^{18}. The results of this study are shown in table 7. When O^{18}-formate was incubated in the complete system with ATP and THF, it was found that the O^{18} had exchanged completely with the oxygen of orthophosphate, indicating the formation of a bond between these two reaction components.

In summary, a reaction mechanism for the THF-formylase reaction based on the results presented is shown in figure 10. An initial reaction involving the binding of ATP with the enzyme is proposed. The enzyme bound ATP then undergoes a concerted reaction in the presence of formic acid and THF, initiated by a nucleophilic attack by the nitrogen atom on the formic acid, to form the transient intermediate shown, which, after the electron shifts indicated, finally yields the products: orthophosphate, ADP and 10-formyl-THF. This reaction results in the direct transfer of O^{18} from formate to phosphate. In the absence of the reaction substrates, the enzyme bound ATP may be so activated that added ADP may exchange with the enzyme bound ADP moiety. If this reaction were much slower than the reactions occurring with THF and HCOOH, it would be possible to explain the relative slowness of the ADP-ATP exchange reaction observed in the absence of the other reaction substrates.

It is hoped that with the stoichiometric amounts of the enzyme available, it will be possible to obtain evidence for the enzyme-ATP complex, and that the results obtained with this highly purified enzyme will be of value in elucidating the reaction mechanisms of the group of so-called 'enigmatic ATP-reactions.'

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SYMPOSIUM ON BIOCHEMICAL LESIONS OF CARBOHYDRATE METABOLISM

Genetic and hormonal control of glycogen metabolism[1]

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In recent years there has been considerable interest in understanding the role of the various enzymes in metabolic activities of the intact cell. The enzymes of glycogen synthesis and breakdown will be considered in these terms in the present review. Much information has been derived from studies of the hormonal control of glycogen metabolism, as well as from studies of the diseases of glycogen storage. These studies, based on the framework of the structure of glycogen and of the biochemical characteristics of the isolated enzymes, have led to important advances in our knowledge in this area.

GLYCOGEN STRUCTURE

Chemical and physical studies have shown that glycogen is a branched polymer of glucose containing 1,4 and 1,6 linkages. The molecular arrangement of the 1,6 linked glucose residues indicated in figure 1 as black dots, constituting somewhat under 10% of the total glucose residues has been studied by enzymic methods (11). Such experiments have indicated that the 1,6 or branch linkages are arranged in an irregular, rather than a regular branched structure (15). The arrangement is such that the number of 1,6 linkages in the tiers or layers susceptible to successive enzymic attack decreases as the reducing or aldehyde end of the polymer is approached. Recent chemical studies, in which nigerose and isomaltotriose have been isolated in even smaller amounts than isomaltose, indicate the presence of 1,3 and double 1,6 linkages in some glycogen samples (33, 34). Further work will be required to assess the significance of these linkages and the enzymic pathways of their synthesis and breakdown.

[1] Supported in part by grants from the Cleveland Diabetic Fund, the Fund for the Study of Diabetes and Related Metabolic Disorders, and from U. S. Public Health Service (A-2366).

Physical studies by ultracentrifugation techniques have indicated that glycogen isolated by a number of procedures has not a single molecular size but rather a wide spread range of distribution of molecular sizes (16). As shown in figure 2, rabbit liver glycogen isolated by alkaline digestion procedures had a range of molecular sizes from under 1 million up to 190 million. After digestion with crystalline muscle phosphorylase, the upper range of sizes had decreased to 40 and 50 million depending on reaction conditions. That phosphorylase acts differentially with regard to molecular size in degrading glycogen is evidenced by such experiments which indicate that the high molecular weight glycogen species of alkali isolated glycogen are preferentially degraded by the enzyme (16). In the direction of glycogen synthesis, Stetten and Stetten (26) have concluded that both liver and muscle phosphorylases *in vitro* show differential reactivities with glycogens of differing molecular weights. In view of the demonstration of an *in vivo* differential molecular size labelling of liver and muscle glycogen (26) detailed studies of the influence of molecular size of glycogen on the kinetics of glycogen synthesis with UDPG glycogen transglucosylase would be of considerable interest. Since the molecular size distribution is present following a number of different isolation procedures, it probably reflects, in terms of molecular size, a composite of the action of the enzymes of glycogen synthesis and breakdown at the time of isolation.

GLYCOGEN ENZYMOLOGY

In 1936 the Coris isolated glucose-1-phosphate from a reaction mixture containing muscle extract, glycogen, inorganic phosphate and adenylic acid to activate the phosphorylase (3). Subsequent work has shown that this enzyme exists in muscle in two interconvertible forms, one active in the absence of adenylic acid, and the other

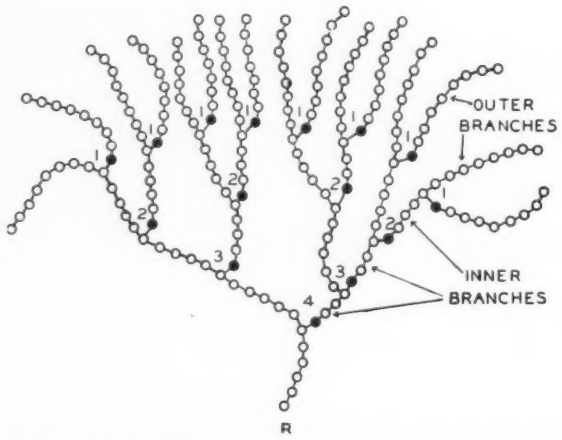

FIG. 1. Model of glycogen structure (15). Numbers refer to tiers or layers of branchpoints susceptible to successive enzymic attack.

requiring adenylic acid for activity; the so called A and B forms, respectively (5). Based on the recognition of the 1,4 linkage specificity of highly recrystallized muscle phosphorylase, came the separation and identification of amylo-1,6-glucosidase, the debranching enzyme (7), and subsequently of amylo 1,4 → 1,6 transglucosidase, the branching enzyme (14). These enzymes are listed in table 1. The action of the debranching enzyme is hydrolytic, while the branching enzyme acts by a transfer reaction substituting 1,6 for 1,4 linkages; in this latter reaction the basal segment of an outer chain is converted to an inner chain during branching. Two recent additional enzymes of the uridine linked pathway are also listed (32): UDPG glycogen transglucosylase, discovered by Leloir and Cardini (19) and UDPG pyrophosphorylase (12). Enzymes with corresponding actions found in plant sources are also indicated.[2]

IN VIVO SIGNIFICANCE OF ENZYMES METABOLIZING 1,6 LINKAGES

The problem of the intracellular or *in vivo* significance of the enzymes of glycogen metabolism may be considered in terms of the 1,6 linkages, and the 1,4 linkages, respectively. Cori and co-workers (10) and Hers (8) have studied a class of glycogen storage disease (Type 3) with an enzymic deficiency of debranching enzyme. Such studies are shown in table 2. In this table data from three such cases numbered *1*, *7*, and *6* are contrasted with findings from a generalized storage disease in which no deficiency of debranching enzyme is present (*case 2*). It may be noted that associated with the deficient (*case 7*) or absent glucosidase (*case 6*) glycogen was isolated with shorter than normal outer chains as measured by phosphorylase degradation, with a corresponding increased percentage of 1,6 linkages. In one (*case 6*) outer chains were sufficiently short so as to approach the completely degraded state, the limit dextrin. Only 3% additional glucose was removed from the glycogen by phosphorylase degradation, as compared with 42% in *case 2* with no glucosidase deficiency. That glycogen with short outer chains is not a necessary feature of this disease is indicated by *case 1* where decreased glucosidase activity is associated with a normal outer chain length of isolated glycogen. The variation in outer chain length with debranching enzyme deficiency may be explained by the nutritional state at time of isolation. The abnormality of structure would be expected to be influenced by nutritional state and show up only under conditions in which glycogen reserves were being drawn upon for energy purposes. The enzyme defect was in keeping with the abnormal glycogen structure. This work demonstrated that an increased tissue content of glycogen was associated with a deficiency of an enzyme concerned with glycogen breakdown and pointed out the importance of amylo-1,6-glucosidase in glycogen metabolism.

The *in vivo* significance of the branching enzyme was indicated by the work of Stetten and Stetten (27) who traced the radioactivity of labelled glucose into glycogen. It was shown that following a single injection of labelled glucose (fig. 3), glycogen outer chains (black dots) were labelled after 3 hours with higher specific activity than inner chains (white dots). These results are plotted in figure 3 in terms of percentage of total counts. Both in liver glycogen or in carcass glycogen (chiefly muscle) the radioactivity of outer chains decreased during the 48-hour period of the experiment, while radioactivity of inner chains increased. At 48 hours radioactivity of inner chains of carcass glycogen approached that of outer chains, or in the case of liver, radioactivity of inner chains exceeded that of outer chains. It was concluded from these and similar experiments that glucose residues in outer chains of glycogen molecules were renewed at a faster rate than those situated internally. Further, labelled glucose residues initially located in outer chains, with time found their way into more centrally located layers. These experiments were consistent with the action of branching enzyme which acts to convert outer chains to inner through a branching reaction. Decreased radioactivity of outer chains with time would then be explained by resynthesis of branched outer chains with unlabelled glucose residues, since radioactivity of blood glucose would decrease during the course of the experiment. These two types of studies pointed up the intracellular importance of debranching and branching enzymes in glycogen metabolism.

IN VIVO SIGNIFICANCE OF ENZYMES METABOLIZING 1,4 LINKAGES

The problem of the synthesis and breakdown of the 1,4 linkages was opened largely by the work of Sutherland (28). He demonstrated that glycogen breakdown subsequent to epinephrine or glucagon treatment was associated with increased phosphorylase activity. This find-

[2] Since the metabolic roles of the tissue amylases and transglucosylases have not been elucidated, these enzymes have not been included in the present discussion.

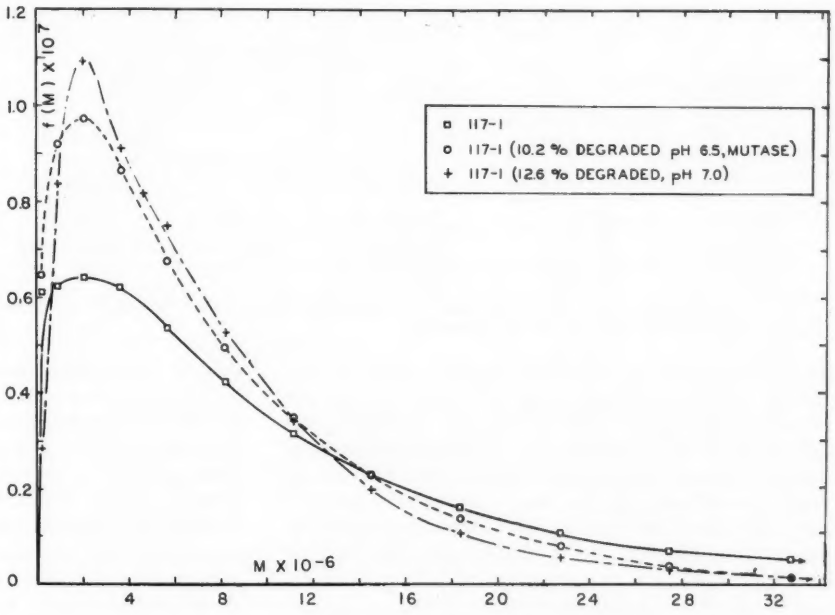

FIG. 2. Molecular weight distributions of rabbit liver glycogen before and after phosphorylase degradation (16).

ing was difficult to interpret in the absence of additional information, but further experiments indicated its general physiological significance. It was subsequently demonstrated by Krebs and Fischer (13) that in resting muscle, the inactive or B form of the enzyme predominated. Cori and Illingworth (6) confirmed this observation. They further showed that with muscular contraction the breakdown of glycogen was linked with increased phosphorylase activity. In recent work, Robbins, Traut and Lipmann (23) demonstrated this association in broken cell preparations. In muscle homogenates activation of phosphorylase by addition of purified kinase and ATP was associated with glycogen breakdown. Thus the breakdown of glycogen with hormone action, or muscular contraction, was associated with increased phosphorylase activity; and in the other sense activation of phosphorylase in muscle homogenates was linked with a breakdown of glycogen.

Another aspect of this problem came to our attention in work with insulin stimulated glycogen synthesis in rat diaphragm (18). As shown in table 3, after a 10 minute incubation period at 38° there was observed the well documented increased tissue glycogen content of diaphragms incubated with insulin, as well as the increased uptake of glucose. Over 90% of the increased

TABLE 1. *Polysaccharide Metabolic Enzymes*

Animal	Plant
Amylo-1,6-glucosidase (debranching enzyme)	R-enzyme
Amylo-1,4 → 1,6-transglucosidase (branching enzyme)	Q-enzyme
Phosphorylase	P-enzyme
UDPG pyrophosphorylase	UDPG pyrophosphorylase
UDPG glycogen transglucosylase	

TABLE 2. *Glycogen Content, Structure, and Enzymes in General Glycogen Storage Disease* (10)

Case No.	Tissue	Glycogen Content	1,6 Bonds in Glycogen	Degradation of Glycogen by Phosphorylase	Glucosidase Activity
		%	%	%	*U*/100 *ml*
2	Heart	8.2	7.7	42	102
1	Psoas	12.7	8.1	41	17
7	Diaphragm	8.9	9.6	26	6
6	Diaphragm	5.1	12.3	3	None

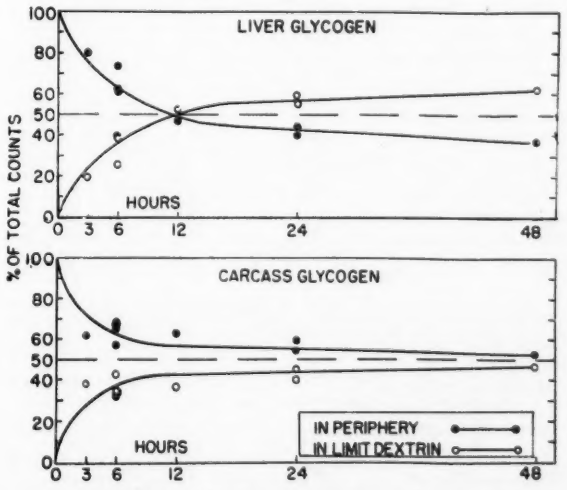

FIG. 3. Distribution of radioactivity in liver and carcass glycogen between periphery and limit dextrin (27).

TABLE 3. *Medium Glucose Uptake and Glycogen Formation by Control and Insulin-Stimulated Rat Diaphragms (See ref. 18)*

Medium glucose uptake

Medium Glucose Conc., *mg/100 ml*	Control, *mg/gm/10 min*	Insulin, *mg/gm/10 min*	Mean Dif., *mg/gm/10 min*
140	1.43 (13)*	1.82 (13)	.39±.027
280	1.95 (13)	2.41 (13)	.46±.049

Tissue glycogen content

Perchloric Acid Soluble			Residual			Glycogen Yield %
Control, *mg/gm*	Insulin *mg/gm*	Mean Dif., *mg/gm/10 min.*	Control, *mg/gm*	Insulin, *mg/gm*	Mean Dif., *mg/gm/10 min.*	
.814 (10)	1.172 (10)	.358±.053	1.641 (9)	1.687 (9)	.046±.034	92
.744 (8)	1.073 (8)	.299±.062	1.438 (9)	1.560 (9)	.122±.038	92

* No. of experiments indicated by the figure in parentheses. Each experiment is the mean of 6 control and 6 insulin treated vessels, each vessel containing two hemidiaphragms.

uptake of glucose in these experiments could be accounted for by increased tissue glycogen content. In previous experiments with 30 minute incubations a yield of about 75% was reported (1). Studies of the acid soluble phosphate intermediates were undertaken and of special interest were the glucose-1-phosphate determinations. As shown in table 4, only small amounts were present from $2.8–5 \times 10^{-5}$ M under these experimental conditions. Levels of inorganic phosphate approached 0.01 M. Ratios of inorganic phosphate/glucose-1-phosphate of 250 and 305 were observed as estimated by chemical and enzymic methods (18). In another organism, yeast, which forms glycogen, similar measurements by Trevellyan, Mann and Harrison (29) indicated a large excess of inorganic phosphate over glucose-1-phosphate. It appeared that either phosphorylase was synthesizing glycogen under unusual local conditions or, alternatively, synthesis might be taking place by another reaction bypassing inorganic phosphate.

In 1957, Leloir and Cardini (19) discovered an enzyme catalyzing the conversion of UDPG to glycogen in liver. Subsequent work demonstrated the presence in muscle not only of this enzyme, but, in addition, the necessary substrate forming enzyme UDPG pyrophosphorylase. These two enzymes together constitute a reaction sequence from glucose-1-phosphate to glycogen with pyrophosphate as product (32). Reversibility has not been demonstrated with UDPG glycogen transglucosylase in contrast to phosphorylase, suggesting that the equilibrium of the UDPG reaction at neutral pH is displaced toward glycogen synthesis. The UDPG reaction differs from the phosphorylase reaction in that the condensing monomer is a doubly substituted phosphate derivative of glucose (UDPG) in contrast to a singly substituted phosphorylated glucose (glucose-1-phosphate).

TABLE 4. *Glucose-1-Phosphate Contents of Control and Insulin-Stimulated Rat Diaphragms* (18)

Medium Glucose Con., *mg/100 ml*	Glucose-1-Phosphate	
	Control, *μmoles/gm*	Insulin, *μmoles/gm*
140	0.034 (6)*	0.028 (6)
280	0.048 (8)	0.050 (8)
Ratio P/G-1-P	$\frac{8.49}{0.034} = 250$	$\frac{8.55}{0.028} = 305$

* No of experiments indicated by the figure in parentheses.

Thus it was shown that there existed in muscle two systems capable of synthesizing 1,4 linkages of glycogen; the uridine linked pathway acting via UDPG and phosphorylase utilizing glucose-1-phosphate. The physiologic significance of the uridine pathway assumed importance through studies of patients with a myopathy originally described by McArdle (21). This disease was delineated clinically as one of impaired glycogen breakdown because of failure of vigorous exercise to evoke an elevation of blood lactic acid. Subsequent biochemical studies established an elevated muscle glycogen content (24). Enzymic studies by several groups (17, 22, 25) have shown a specific defect in phosphorylase with no defect in UDPG pyrophosphorylase, UDPG glycogen transglucosylase, amylo-1,6-glucosidase, and phosphoglucomutase. Determinations, as shown in table 5, have yielded a value of total phosphorylase of 0.23% as compared to 2 controls of the same age group.

Thus the two diseases, Type 3 storage disease and McArdle's disease, may be contrasted and shown each to be associated with a storage of glycogen. In one case an enzyme degrading 1,6-linkages is defective, and in the other an enzyme breaking down 1,4 linkages. In both cases the defect appears to be specific, inasmuch as the enzyme acting on the other linkage type has been demonstrated to be present. A summary of the currently delineated types of diseases associated with a storage of glycogen is shown in table 6. A similar summary has been previously presented by Cori in her Harvey Lecture (2).

From the experiments with McArdle's disease, it was concluded that glycogen could be synthesized in a muscle deficient in phosphorylase. The evidence outlined was consistent with separate enzymic pathways for synthesis

TABLE 5. *Enzyme Activities in Muscle* (17)

Patient	Amylo-1,6-Glucosidase	Phosphorylase	UDPG Pyrophosphorylase	Mutase
	per 1000/ mg/hr.	*μm/min./g tissue*	*μm/min./g tissue*	*μm/min./g tissue*
1 McArdle's disease	.006	0.025	28.6	63.4
2 Control	.004	9.20	32.0	67.5
3 Control	.010	10.65	26.3	56.4

TABLE 6. *Types of Glycogen Storage Diseases*

Disease	Organ Distribution	Enzyme Defect	Glycogen Structure	Remarks	Reference
Von Gierke (Type 1)	liver kidney	glucose - 6 - phosphatase	normal	defect of enzyme not directly involved in glycogen metabolism	(4)
McArdle	muscle	phosphorylase	normal	defect of enzyme of glycogen breakdown	(17, 22, 25)
Liver phosphorylase deficiency	liver	phosphorylase	not studied	defect of enzyme of glycogen breakdown	(8)
Type 3	generalized	amylo - 1,6 - glucosidase	abnormal or normal	defect of enzyme of glycogen breakdown	(8, 10)
Type 4	generalized	? amylo-1,4 → 1,6 - transglucosidase*	abnormal	? defect of enzyme of glycogen synthesis	(2, 9)
Type 2	generalized	unknown	normal		(2)

* Suggested by abnormal glycogen structure.

and breakdown of 1,4 linkages of glycogen, synthesis by the uridine linked pathway through UDPG and degradation by phosphorylase. As shown in figure 4, summation of this series of reactions would lead to a cyclic reaction sequence with a junction point at the level of glucose-1-phosphate.

Two additional recent studies add further evidence to the physiological importance of the uridine pathway

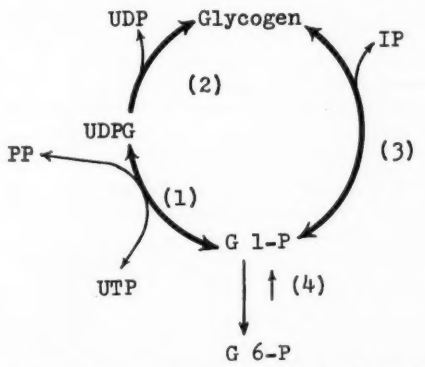

FIG. 4. Enzymes of the glycogen cycle (30). (1) UDPG pyrophosphorylase; (2) UDPG glycogen transglucosylase; (3) phosphorylase; (4) phosphoglucomutase.

TABLE 7. *Glycogen Content and Enzyme Activities of the Glycogen Cycle in Rat Tissues* (30)

Tissue	Fed Glycogen Content	Mutase	UDPG Pyrophosphorylase	UDPG Glycogen Transglucosylase	Phosphorylase
	mg/g tissue	*μm/hr./g tissue*	*μm/hr./g tissue*	*μm/hr./g tissue*	*μm/hr./g tissue*
Muscle	4.0	6670	640	220	1820
Liver	20.2	2240	3540	187	300
Heart	1.68	600	227	166	144
Kidney	0.87	506	203	31	31
Spleen	1.15	154	221	37	22
Lung	0.83	226	183	36	56
Brain	1.96	216	88	32	38
Testis	0.24	98	97		5
Intestinal mucosa	0.85	34	114		0.1
Fat	0.21	42	12		0.8

TABLE 8. *Phosphoglucomutase, UDPG Pyrophosphorylase and Phosphorylase Activities of Extracts of Control and Insulin-Stimulated Diaphragms* (31)

	Control μm/g/hr.	Insulin, μm/g/hr.
Phosphoglucomutase	7484	7671
UDPG pyrophosphorylase	440	437
Phosphorylase, total	2262	2364

TABLE 9. *UDPG-Glycogen Transglucosylase Activities of Control and Insulin-Stimulated Diaphragms* (31)*

	Control, μm/g/hr.	Insulin, μm/g/hr.	Difference	P
No G-6-P in test	13.2	18.0	4.8±0.5	<0.001
G-6-P added	59.8	58.4	1.4±3.4	>0.50

* Uncorrected for self absorption of radioactivity in presence of glycogen.

in glycogen synthesis. Studies of the tissue distribution of these enzymes (30) demonstrate that they are widespread in a number of organs (table 7) and tissues, and there appears to be a general relationship between fed glycogen content and the levels of activity of these enzymes. Data of UDPG glycogen transglucosylase are from Leloir and co-workers (20). Phosphoglucomutase has been found in general to be present in excess over the activities of the other enzymes studied. UDPG pyrophosphorylase appears also in most tissues to be non-rate-limiting. The case of liver, high UDPG pyrophosphorylase activity, would be in keeping with its general biosynthetic role for UDPG products in addition to glycogen. In most tissues rate-limiting enzymes appear to be phosphorylase and UDPG glycogen transglucosylase present at comparable levels of activity. In muscle total phosphorylase activity is higher than UDPG glycogen transglucosylase. As previously mentioned, in resting muscle phosphorylase is present predominantly in the inactive or B form. As such it would permit the accumulation of glycogen by the enzymes of the UDPG pathway. In exercise, or under the action of epinephrine, phosphorylase would become active and provide the muscle with a rapid supply of hexose phosphate as energy source from glycogen breakdown.[3]

[3] It is to be stressed that the data presented are not sufficiently quantitative to rule out additional enzymatic reactions.

A second evidence has recently been uncovered which points to the physiological significance of the uridine pathway in glycogen synthesis and implicating this enzyme in the hormonal control of glycogen metabolism by insulin (31). Measurements of the four enzymic activities in extracts of diaphragms preincubated without and with insulin were made. As shown in table 8, activities of phosphoglucomutase, UDPG pyrophosphorylase and phosphorylase did not differ significantly. Increased activity of UDPG glycogen transglucosylase (table 9) was observed and of sufficient magnitude to account for increased glycogen content in the presence of insulin. Enzymic activity was found to be increased in extracts prepared from diaphragms incubated with insulin as compared with controls. That this increase is not due to soluble glucose-6-phosphate or other soluble activator is shown by several controls including ammonium sulfate fractionation of extracts, dialysis, and addition of glucose-6-phosphate to extracts, precipitation of the enzyme with ammonium sulfate with washing out of this cofactor in the supernatant fluid. Since activity in presence of excess glucose-6-phosphate is unchanged while activity in absence of glucose-6-phosphate is increased, this experiment suggests the existence of two forms of the enzyme, one active in absence of glucose-6-phosphate and the other requiring this cofactor for activity. The insulin mediated effect could represent an interconversion of these two forms. While it is too early to ascertain the mechanism of this effect or its significance with regard to insulin action, it appears to us to point to the physiologic importance of the synthetic capacity of the uridine pathway of glycogen synthesis.

SUMMARY AND CONCLUSIONS

It would appear, in conclusion, that the synthesis and breakdown of both 1,6 and 1,4 linkages of glycogen is brought about by separate enzymes, and that these enzymes function intracellularly in the metabolism of glycogen. Because of the coexistence of separate enzymic systems capable of glycogen synthesis in the cell it would appear that cells sacrifice simplicity for other purposes including hormonal control.

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Essential pentosuria and the glucuronate-xylulose pathway[1]

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THE STUDY OF HARMFUL, hereditary metabolic defects of man may be based on a hope that the discovery of the cause of the defect will lead to improvement in the clinical management of the afflicted. This motive is necessarily absent in the case of those defects which appear to have no deleterious effect on the health of the persons involved. However, just as bacterial mutants have provided important clues to metabolic systems, the study of humans with apparently harmless abnormalities can also lead to the discovery of hitherto unknown pathways of metabolism. Essential pentosuria has served such a role. Its study has yielded information about the metabolism of normal mammals, although the relative importance of this new knowledge has not yet been established.

Before presenting the results and discussing the implications of comparatively recent investigations of this disorder, I should like to review briefly the fundamental work which is the basis of its characterization as an inborn error of metabolism. (For a fuller discussion of certain aspects, a review (53) may be consulted.) The detection, by Salkowski and Jastrowitz (51), of a pentose in urine in 1892 is noteworthy not only because it was the recognition of the first case of pentosuria but also because it marked the discovery of pentose in material of animal origin. It was 2 years later that ribose was discovered in pancreatic extracts (29).

NATURE OF THE URINARY SUGAR

Considerable confusion existed for many years as to the identity of the urinary pentose, largely because some rather extensive chemical studies led to the conclusion that it was racemic arabinose. The turning point occurred in 1913, when Zerner and Waltuch (64, 65) prepared L-xylose phenylosazone from the urine of a patient with hereditary pentosuria. One year later, Levene and La Forge (47) showed that the pentose is a ketose, not an aldose as had previously been assumed, thus proving that the sugar is L-xylulose (originally designated *d*-xyloketose). Since 1920, all authenticated cases of essential pentosuria have been shown to involve L-xylulose, although other pentoses may be excreted in alimentary pentosuria.

GENETICS OF THE DISORDER

The recognition that pentosuria has a familial basis occurred long before the urinary sugar was properly characterized. In 1906, Janeway (43) wrote that pentosuria is 'rather analogous to cystinuria and alkaptonuria than to diabetes,' and in 1908, Sir Archibald Garrod (26) designated it as one of his 'inborn errors of metabolism.' As the detection of pentosuria became more frequent, it became evident that authenticated cases of L-xylulose excretion were limited to persons of Jewish origin, with an incidence of perhaps one in a few thousand (53). The most complete and rigorous study of the genetics of the disorder was made by Lasker, Enklewitz, and Lasker (46) who concluded that the defect is transmitted as an autosomal, recessive Mendelian trait. In 1953 there appeared the first report, by Barnes and Bloomberg (4), of the occurrence of essential pentosuria in non-Jewish individuals, namely, in two Lebanese sisters. Very recently, Khachadurian (private communication), at the American University of Beirut, has found six cases of the disorder in a highly inbred Arab family. Although there is apparently no sex linkage of the trait among Jews (46), the defect was found exclusively in males in this Lebanese family. Further investigation of this point seems warranted.

It should be emphasized that there is no reliable scientific or clinical basis for believing that pentosuric individuals are at any disadvantage in regard to their state of health. There is no difference in longevity between pentosuric and nonpentosuric individuals (45). For the time being, pentosuria should be regarded not as a disease, but simply as a metabolic disorder.

ORIGIN OF L-XYLULOSE

Between the early chemical work on the identification of the urinary sugar and the metabolic investigations of the last few years, there are the classical investigations of Enklewitz and Lasker (22), whose biochemical insight and experimentation provided the basis for the more recent definitive studies on the origin of L-xylulose, studies made possible by the availability of tracer and enzymatic techniques. In brief, these two investigators reasoned that drugs, such as aminopyrine, which were already known to increase xylulose excretion in pentosurics, exerted their effect by stimulating the metabolic system by which glucuronides are produced. In support of this

[1] Investigations in the author's laboratory were supported in part by the National Science Foundation and the Howard Hughes Medical Institute.

hypothesis they found that borneol and menthol, both glucuronogenic compounds, stimulated xylulose excretion. Finally, and most important, they tested glucuronic acid, as the lactone, and observed a marked enhancement of xylulose excretion by pentosuric subjects, although no effect was observed in a normal subject. The mechanism by which the various drugs exert their effect is obscure, but the metabolic relationship of glucuronic acid to xylulose is now clear.

Several years ago we decided to study pentosuria by approaches which had been successful with other inborn errors of metabolism. L-Xylulose excretion by the pentosuric seemed to be offering a clue to an unknown facet of mammalian metabolism. We were fortunate in being able to elicit the cooperation of a pentosuric medical student at Vanderbilt. The influence of D-glucuronolactone on the daily output of xylulose by this subject is shown in figure 1. The negligible effect of sodium glucuronate is undoubtedly due to the low permeability of this form of the uronic acid. Note the rather constant amount of 2 g of xylulose excreted daily, except when glucuronolactone was ingested. When very large amounts of glucuronolactone were administered to nonpentosuric humans and to guinea pigs, it was possible to isolate, by chromatographic methods, milligram quantities of the pentose. As our methods improved, we were able to isolate xylulose even without the administration of the uronic acid (55, 56). Futterman and Roe (25) also reported the isolation of L-xylulose from the urine of normal humans and from rat urine as well. L-xylulose could thereafter nc longer be considered to be a unique product of individuals with the hereditary abnormality.

The mechanism by which D-glucuronolactone enhances xylulose excretion was the next facet placed under study. Although it was obvious from structural considerations that other stimulators of xylulose excretion could not serve as direct precursors, it appeared likely that glucuronic acid could be converted to L-xylulose. The postulation of mechanisms for this conversion (56) was based upon knowledge of the enzymatic oxidative decarboxylation of 6-phospho-D-gluconate to D-ribulose-5-phosphate (38) and upon the roles of D-glucuronic acid and L-gulonic acid as precursors of L-ascorbic acid (39, 40). The synthesis of C^{13}-labeled glucuronolactone by the Corn Products Refining Company, and the generous donation of this material by Dr. Neal A. Artz, made possible the appropriate experiments in the pentosuric human.

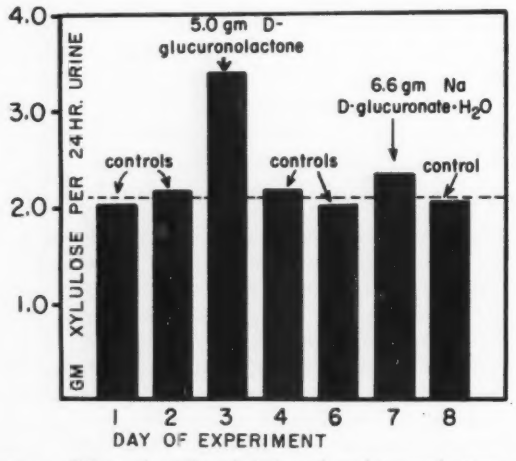

FIG. 1. Effect of orally administered D-glucuronolactone on L-xylulose excretion by a pentosuric subject (56).

The mechanism of the conversion of D-glucuronolactone into L-xylulose is shown in figure 2. The over-all mechanism was demonstrated by the C^{13} studies (57). When D-glucuronolactone-6-C^{13} was administered orally to our subject, the isolated urinary L-xylulose did not contain excess C^{13}. This result is consistent with the hypothesis that the carboxyl carbon is lost through the decarboxylation of the intermediate β-keto acid. On the other hand, D-glucuronolactone-1-C^{13} yielded urinary pentose labeled primarily in the C_5 position. Evidence for a direct conversion of glucuronate to xylulose in animals was also obtained in extensive enzymatic studies in several laboratories, leading to the conclusion that L-gulonate is a common intermediate in the biosynthesis of both L-xylulose and L-ascorbic acid (8, 13, 28, 41, 60). The situation has been greatly complicated, however, by the existence of lactonases in tissues, which has led to difficulties in determining the relative importance of lactones and salt forms of the sugar acids (21, 61–63). The various transformations which have been demonstrated for D-glucuronic acid, L-gulonic acid, and their lactones are shown in figure 3. The direct formation of L-gulonolactone by reduction of D-glucuronolactone has only recently been reported (49). Perhaps the main point to note is that whereas L-gulonolactone is oxidized, through a 2-keto derivative, to form ascorbic acid, L-gulonate is the substrate for the L-xylulose-forming enzyme (2, 9, 44).

CONVERSION OF L-XYLULOSE TO XYLITOL AND D-XYLULOSE

The next major problem in considering the metabolic role of L-xylulose involves its utilization in normal mammals. It is obvious that if pentosuria is analogous to other inborn errors of metabolism in being characterized by the accumulation of a normal metabolic intermediate, there must be, in normal animals, an enzymatic system to dispose of the pentose. This was suggested by some *in vivo* experiments done many years ago, and is supported

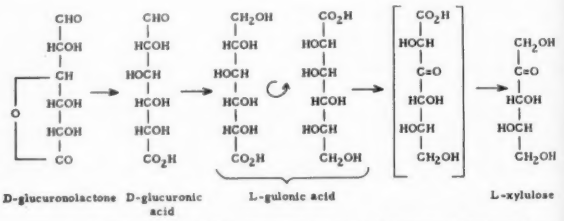

FIG. 2. Mechanism of the conversion of D-glucuronolactone into L-xylulose.

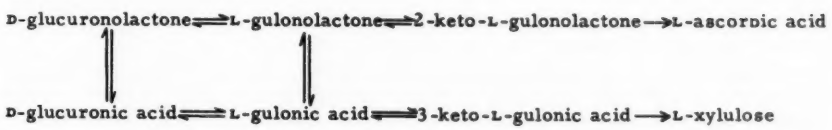

FIG. 3. Reactions of D-glucuronic acid and L-gulonic acid and their lactones.

by the fact that only a little xylulose is excreted by normal individuals given massive doses of glucuronolactone (56).

When L-xylulose is incubated with guinea pig liver slices or homogenates, rather rapid utilization of the pentose occurs (55, 59). By differential centrifugation, the various subcellular fractions were prepared and then tested for the presence of the xylulose-metabolizing system. It was found to occur only in the mitochondrial fraction (59). Analysis of incubation mixtures showed there was no accumulation of expected products such as sugars or their phosphate esters, but since there was no decrease in periodate-reactive material as the enzymatic reaction proceeded, it seemed likely that the product was a polyhydric alcohol. The structures of the two polyols which could be formed directly from L-xylulose are shown in figure 4. Isolation of the polyol product as the crystalline pentacetate showed that it was xylitol (59). Moreover, xylitol, but not L-arabitol, led to the formation of xylulose when it was incubated with mitochondrial preparations.

The next stage of this work led to the finding that furnished the clue as to the final fate of L-xylulose. When fractions of ruptured mitochondria were tested, the enzyme system responsible for the utilization of the pentose was found in the insoluble, lipoprotein portion (35). Further treatment of this fraction yielded a clear, stable aqueous solution of the enzyme suitable for spectrophotometric experiments. The interconversion of L-xylulose and xylitol was found to be TPN-linked, and the enzyme showed very great substrate specificity. In the same extract, moreover, there was found a second xylitol

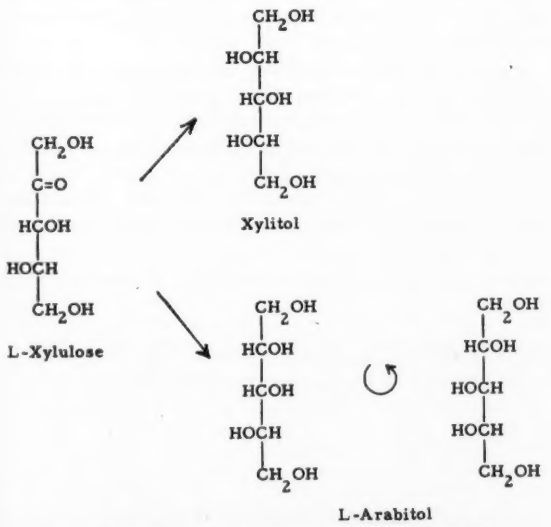

FIG. 4. Reduction of L-xylulose to xylitol or L-arabitol.

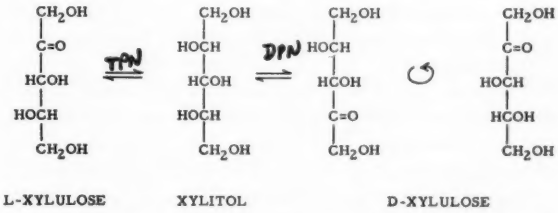

FIG. 5. Enzymatic conversion of L-xylulose to D-xylulose via xylitol.

dehydrogenase, this one linked to DPN and yielding D-xylulose rather than L-xylulose. The two enzymes furnished an enzymatic mechanism for the conversion of L-xylulose to D-xylulose via xylitol (35, 36), as shown in figure 5. These enzymes have been separated and purified by Hickman and Ashwell (32) and by Hollmann (34). The TPN[2]-linked enzyme, best referred to as TPN-xylitol (L-xylulose) dehydrogenase, shows appreciable activity only towards L-xylulose and xylitol among many compounds tested, but the DPN-linked enzyme acts on a considerable number of ketoses and polyols. The latter enzyme has not been successfully separated into fractions active towards different substrates. It resembles closely, but is not identical to, the non-particulate DPN-linked polyol dehydrogenase of liver first studied by Blakley (6). However, the fact that, with the mitochondrial enzyme, xylitol has the lowest Km value of all the polyol substrates lends support to the view that this pentitol is the physiological substrate (34).

GLUCURONATE-XYLULOSE PATHWAY

The discovery of the interconversion of xylitol and D-xylulose at once raised the possibility that D-glucuronate, L-gulonate, and L-xylulose are metabolized via the pentose phosphate pathway, in which D-xylulose-5-phosphate is a key intermediate. The formulation of

[2] The following abbreviations are used in this paper.

AA: ascorbic acid
ATP: adenosine triphosphate
DPN, DPNH: diphosphopyridine nucleotide and its reduced form
G-1-P: glucose 1-phosphate
G-6-P: glucose 6-phosphate
GA: glucuronic acid
GA-1-P: glucuronic acid 1-phosphate
Gul. A.: gulonic acid
Gul. L.: gulonolactone
TPN, TPNH: triphosphopyridine nucleotide and its reduced form
UDPG: uridine diphosphate glucose
UDPGA: uridine diphosphate glucuronic acid
UDPGal: uridine diphosphate galactose
Xu: xylulose

such a pathway (35, 36), being based primarily on enzymatic data, required support from experiments in intact animals and also the direct demonstration of the existence of a D-xylulokinase. This enzyme was reported by Hickman and Ashwell (33) to occur in calf liver, thus completing the enzymatic basis of the pathway.

The tracer experiments designed to check the pathway *in vivo* have been based upon the work of Horecker *et al.* (37), who showed that a C_1-labeled pentose entering the pentose phosphate pathway without prior chain cleavage labels liver glycogen in the C_1 and C_3 positions. In our laboratory, McCormick (50) determined the labeling pattern in glycogen after the administration of xylitol-1-C^{14} (prepared from D-xylose-1-C^{14}), obtaining the results shown in table 1. Eisenberg, Dayton, and Burns (11, 20), in a similar manner, checked a longer portion of the pathway by administering suitably labeled D-glucuronolactone and L-gulonolactone. It thus appears that the cycle shown in figure 6 exists in mammalian liver. The role of the enzyme which can reduce D-glucuronolactone to L-gulonolactone without L-gulonate as an intermediate remains to be determined. Moreover, the mechanism by which glucose or glucose phosphate is converted into D-glucuronate is still not firmly established. Finally, there are remarkable catalytic effects of certain compounds on the pathway which suggest that interesting problems regarding the cycle still await solution. I should like to discuss briefly some of these matters before concluding with an appraisal of our knowledge regarding the specific defect in essential pentosuria.

UDPG PATHWAY AND DRUG EFFECTS

I have already mentioned the stimulation of L-xylulose excretion by various substances administered to pentosuric subjects. Two of these, antipyrine and aminopyrine, are among the large number of noncarbohydrate compounds which were reported by Longenecker, Fricke, and King (48) in 1940 to enhance tremendously the excretion of L-ascorbic acid by the rat. It might be surmised that, since L-xylulose and L-ascorbic acid have common precursors, the effects on the formation of the two L-sugars might have a common mechanism. That this is not so is indicated by the report that borneol has no effect on ascorbic acid production in the rat (12), although it stimulates L-xylulose excretion by the pentosuric. Some comparative data are given in table 2. The remarkable stimulation is perhaps best shown by 3-methylcholanthrene. Conney and Burns (17) have reported that a single 10 mg injection of this hydrocarbon will, after a lag period of 2 or 3 days, cause a rat to excrete many times the normal amount of L-ascorbic acid, the enhancement lasting for several weeks.

TABLE 1. *Distribution of C^{14} in Liver Glycogen After Administration of D-Ribose-1-C^{14} and Xylitol-1-C^{14}* (50)

Substance Injected	Experimental Animal	% Administered C^{14} in Glycogen	Relative % C^{14} of Glucose Carbon Atom No.				
			1	2	3	4+5	6
D-Ribose-1-C^{14} (4.30 μc, 10.4 mg)	Rat ♀ (356 gm)	7.1	69.5	2.4	28.5	2.9	1.4
Xylitol-1-C^{14} (6.70 μc, 10 mg)	Guinea pig ♀ (765 mg)	23.8	67.1	0.4	25.6	2.0	2.1
Xylitol-1-C^{14} (6.70 μc, 10 mg)	Rat ♂ (474 gm)	11.8	67.1	0.6	25.1	2.2	3.3

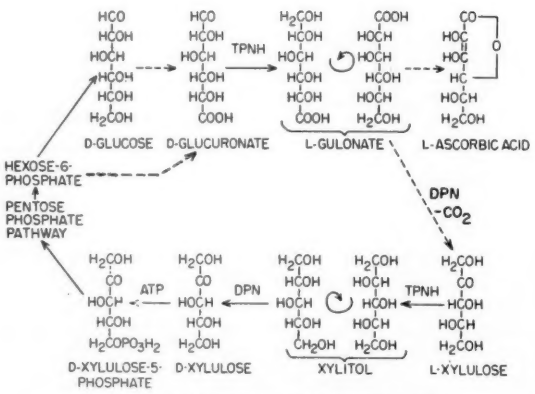

FIG. 6. The glucuronate-xylulose pathway.

Some of the drugs seem to be acting in a manner unrelated to glucuronide formation. For example, an active substance is barbital (12), which is metabolized hardly at all by the rat. The stimulatory effect is not limited to ascorbate but affects the excretion of D-glucuronate and L-gulonate as well. Burns and his group (10, 12) have shown that chloretone and barbital cause increased conversion of labeled glucose to urinary D-glucuronate, L-gulonate, and L-ascorbate, the glucuronate being excreted in unconjugated form. The influence of these drugs is obliterated by hypophysectomy but not by adrenalectomy (12). It is noteworthy that some of the stimulatory agents also enhance many microsomal reactions by a mechanism suggestive of adaptive enzyme formation (17, 18).

Since drug studies involving the estimation of several intermediates have shown that there is increased formation of glucuronate, it appears that the pathway is influenced at a step or at steps preceding this intermediate. Borneol, and presumably menthol as well, however, would appear to be acting through a different mechanism, since L-xylulose formation in the pentosuric is affected but not L-ascorbate production by the rat. The actions of these compounds pose an intriguing pharmacological problem.

TABLE 2. *Effect of Drugs on Excretion of L-Xylulose (L-Xu) in the Pentosuric and L-Ascorbic Acid in the Rat*

Drug*	L-Xu	L-Ascorbic Acid
Antipyrine	↑ (2-fold)†	↑ (20 fold)‡
Aminopyrine	↑ (2-4 fold)†	↑ (20 fold)‡
Borneol	↑ (2 fold)†	O§

* See references for dosage. † From (22). ‡ From (48). § From (12).

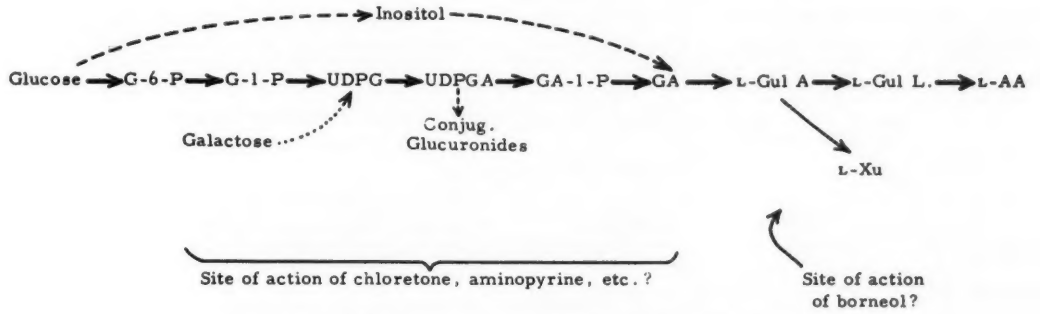

FIG. 7. Origin of D-glucuronate and possible sites of drug action.

Perhaps related to the drug effects is the question as to how the glucuronate originates in the first place. The biosynthesis of uridine diphosphate glucuronic acid is well-established, and Ginsberg *et al.* (27) have shown that two additional enzymatic steps can yield free glucuronate in kidney extracts. An explanation of the drug effects might be that aminopyrine, chloretone, barbital, and other substances act before glucuronate, as shown in figure 7, whereas borneol stimulates only the reaction leading to L-xylulose. Patterson (unpublished observations), in our laboratory, has been unable to detect an appreciable effect of borneol, bornyl glucuronide, or several borneol derivatives on the L-gulonate-L-xylulose transformation, but it is of course still possible that an unknown metabolic product of borneol is the actual agent or that a hormonal mechanism is involved.[3]

It must be mentioned that another potential route to glucuronate is through inositol. Charalampous (16) has found a kidney enzyme which can catalyze the conversion of inositol into D-glucuronate. However, it has been shown that labeled inositol gives rise in the intact rat to labeled glucuronate and L-gulonate but not to L-ascorbate, presumably because the L-gulonate formed in the kidney does not reach the liver cells which synthesize the vitamin (14). In further support of the uridine nucleotide route is the fact that galactose is an even better precursor of ascorbate than glucose (42). The galactose presumably is converted to UDPG via UDPGal.

It is interesting to note the relationships of other metabolic abnormalities to the reactions in figure 7. The galactosemic defect involves an enzyme deficiency between galactose and UDPG, specifically, a lack of galactose-1-phosphate uridyl transferase (23). In addition, in Gilbert's disease (1), in newborn infants having congenital nonhemolytic jaundice with kernicterus (3), and in a mutant strain of Wistar albino rats with constitutional nonhemolytic hyperbilirubinemia (15, 52), there is good evidence that the failure to convert bilirubin to bilirubin glucuronide is due to a deficiency of the enzyme glucuronyl transferase (19). As expected, there is a general failure to use UDPGA to produce conjugated glucuronides. This major progress in our understanding of these conditions was made possible by the elucidation of the chemical basis of the direct and indirect diazo tests for bilirubin (5).

ROLE OF THE CYCLE

In spite of the fascinating effects of the drugs, it is of course possible that the ultimate explanations of the mechanism of their actions will have an importance unrelated to the normal physiological role of the glucuronate pathway. It is apparent that the pathway is useful to the species which have the L-gulonolactone oxidizing system for synthesizing ascorbic acid, but the role of the cycle in other species, such as man, the monkey, and the guinea pig, remains obscure. It is conceivable that it serves in the production of an important sugar whose biosynthesis is still unknown. However, a more promising approach may reside in a consideration of the significance of the large number of oxidation-reduction reactions which take place as the cycle proceeds (fig. 8). TPNH is always used in the reduction of intermediates, whereas DPN is the pyridine nucleotide used for dehydrogenations. It is noteworthy that the concentrations of TPNH and DPN in liver are higher than those of TPN and DPNH, respectively. There is indirect evidence that little carbohydrate is actually metabolized by this route (31). The fact pentosuric individuals are not seriously affected by their disorder indicates that the complete cycle has limited significance under normal circumstances, that compensatory routes are available to the pentosuric, or that reactions other than L-xylulose reduction are important.

THE PENTOSURIC DEFECT

I should now like to return to essential pentosuria to summarize our present understanding of the defect. The

[3] Recent studies are yielding information on the mode of action of the substances that enhance the formation of ascorbic acid. First, induction of protein synthesis is indicated by the blocking by ethionine of the effects of barbital and 3-methylcholanthrene on ascorbic acid excretion by the rat (Touster, O., R. W. Hester, and R. A. Siler, *Biochem. Biophys. Research Communs.* 3: 248, 1960). Second, barbital and chloretone have been found to induce a doubling of the level of UDPG dehydrogenase in rat liver, the barbital effect being prevented by the administration of ethionine (Hollmann, S., and O. Touster, unpublished work). However, two other powerful stimulators of ascorbic acid synthesis, 3-methylcholanthrene and 3,4-benzpyrene, do not affect the activity of this dehydrogenase.

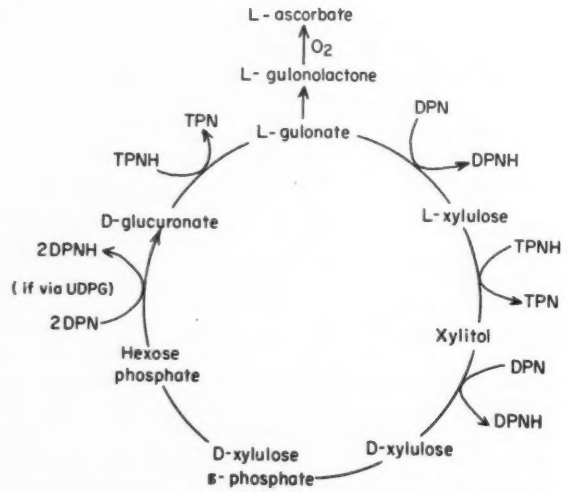

FIG. 8. Oxidation-reductions in the formation of L-ascorbic acid and in the metabolism of L-xylulose.

possibility that the defect is primarily renal is precluded by experiments in which the effect of orally-administered glucuronolactone on the plasma xylulose levels was determined in a pentosuric and in normal individuals (7). The results are shown in table 3. The accumulation of blood xylulose in the pentosuric after the administration of a precursor is consistent with the existence of a metabolic defect but not with a defect in the renal reabsorption of the pentose. Recently, Freedberg *et al.* (24) have reported that carriers of the pentosuric gene give intermediate blood xylulose levels with a glucuronolactone loading test. The ability to detect heterozygotes should be useful in further studies of the genetics of the disorder.

Another question concerns the actual site of the enzymatic defect. Although it is reasonable to assume that there is a deficiency of TPN-xylitol (L-xylulose) dehydrogenase, it occurred to us that a defect between xylitol and D-xylulose was also possible, because the accumulated xylitol would not have been detected by analytical methods based on the reducing properties of sugars. We therefore examined pentosuric urine for the presence of a pentitol, utilizing chromatographic methods and periodate-reactivity as the basis for analysis. A considerable amount of a pentitol was isolated, but it was shown to be L-arabitol rather than xylitol (54). The finding was surprising not only because it was the wrong pentitol, but also because L-arabitol had not previously been found in nature. Since the relatively unspecific DPN-linked polyol dehydrogenase of animal liver has a slow but definite action on L-xylulose (32), perhaps forming L-arabitol, it is possible that the L-arabitol in pentosuric urine is merely a 'detoxication product' of the accumulated ketopentose. The urinary L-arabitol is in fact labeled when a pentosuric is given glucuronolactone-1-C^{13} (54). Mammalian liver has L-arabitol dehydrogenase activity, but various purification procedures do not separate this activity from that shown towards several other substrates of the DPN-linked enzyme (unpublished observation).

TABLE 3. *Effect of 5 g of Oral Glucuronolactone on the Plasma Xylulose-Levels of Normal and Pentosuric Subjects* (7)

Subject	Plasma Xylulose (mg/100 ml)		
	Fasting	1 hr.	2 hr.
Normal (M.K.P.)	<0.3	<0.3	<0.3
Normal (R.C.B.)	<0.3	<0.3	<0.3
Pentosuric (I.B.)	<0.3*	9	8
Pentosuric (I.B.)	2*	7	11

* In two other samples of fasting pentosuric plasma, the xylulose-level was less than 0.3 mg/100 ml.

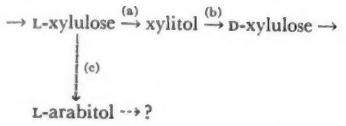

FIG. 9. Site of the pentosuric defect.

TABLE 4. *Urinary L-Xylulose and Ribose in Normal and Pentosuric Patients Given Imidazoleacetic Acid (IAA) and D-Glucuronolactone Uniformly Labeled with C^{14}* (30)

	Normal	Pentosuric
Urinary L-Xu (0–10 hr.) (μmoles)	0.5	3950
% administered C^{14}	0.002*	4.3
Ribose from urinary IAA riboside (0–10 hr.) (μmoles)	275	208
% administered C^{14}	0.058	0.002*

* Significance doubtful because of small quantity of radioactivity.

The relationships are shown in figure 9. The excretion of L-xylulose, but not xylitol, by the pentosuric indicates that step (*a*), rather than step (*b*), is defective. The presumption that step (*c*) is an unimportant one is only tentative. It is based upon the fact that L-arabitol-1-C^{14} yields, in the guinea pig and rat, an unusual labeling pattern in liver glycogen (58) which is inconsistent with the patterns obtained from labeled D-glucuronolactone and with L-gulonolactone.

Hiatt (30) has carried out a tracer experiment which gives some information about the extent of the enzyme deficiency in the pentosuric (table 4). C^{14}-glucuronolactone yields urinary ribose in a normal human, undoubtedly by virtue of the connection between the glucuronate-xylulose cycle and the pentose phosphate pathway. In the pentosuric, however, there is negligible labeling of the ribose, although the urinary xylulose is labeled as expected. It appears, then, that there is a virtual absence of the enzymatic system which can normally act upon the ketopentose.

SUMMARY

It is well-known that pentosuric individuals, being as healthy as the rest of us, do not like to be bothered unnecessarily. We can therefore not expect a donation of

the liver specimen which would make possible the direct demonstration of the absence, or inhibition, of the L-xylulose-xylitol enzyme in individuals with the disorder. However, the evidence for the lack of activity of this enzyme is considerable, and all other possible sites of the abnormality now appear to be eliminated. The more timely and pressing problem involves the explanation for drug effects on the glucuronate-xylulose pathway. And, ultimately, we will learn the significance of the reactions in the normal organism.

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Hereditary defects in galactose metabolism in man and microorganisms[1, 2]

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THE STORY OF GALACTOSE and its role in evolution, in genetics and in general cell physiology would have been most incoherent and unintelligible had it not been for the pioneer work of Kosterlitz and especially that of Leloir. I will for briefness' sake call the pathway to be discussed the Leloir pathway. Leloir found an enzyme, 'galactowaldenase,' which catalyzes the inversion of uridine diphosphoglucose to uridine diphosphogalactose. Dr. Maxwell and I call this enzyme UDPGal-4-epimerase. He showed that DPN is involved in the function of epimerase.

The enzymatic 4-epimerization is essentially a flipping of the 4-hydroxyl group from an equatorial position in glucose to an axial position in galactose (fig. 1). This inversion takes place only if the sugars are esterified by their reducing group with UDP. UDPGlucose is an active compound in the living cell. The arrows in figure 2 indicate some types of fission which have physiological interest. UDPG is, as shown by Leloir (24, 25) and his group, 'active glucose'; UDPGal is, as shown by Gander, Peterson and Boyer (9) and others (3, 5), 'active galactose,' i.e., they are glycosyl donors, the electrophilic group in this case being the 'glycosyl' group (figure 3). We shall not discuss here the various nucleophilic acceptors but just mention one example—the terminal glucose residues (4-hydroxyl group) of glycogen (26).

There are enzymes which catalyze the transfer of the electrophilic 'uridyl' group. These enzymes were described by Munch-Petersen, Smith, Cutolo, and Kalckar some seven or eight years ago. We called them 'uridyl transferases'. One of them, the existence of which was also suspected by Trucco, catalyzes the incorporation of α-glucose-1-phosphate (PG) in a reaction with UTP (16, 30, 31, 37) according to the formula:

$$PG + UrPPP \rightleftarrows PP + UrPPG$$

You could, with Kornberg, call it UDPG pyrophosphorylase; however, because of the existence of a non-pyrophosphorylic fission (see below), we may call it 'PG/PP uridyl transferase.' Munch-Petersen, Braganca and I found, namely, about the same time when we described the above-mentioned uridyl transferase in glucose grown yeast that yeast adapted to galactose synthesizes another uridyl transferase which catalyzes the following reaction (15, 31):

$$PGal + UrPPG = PG + UrPPGal.$$

Logically this enzyme would be called PGal/PG uridyl transferase or abbreviated PGal transferase or in some of the genetics studies we might simply call it 'transferase' or just 'T'. We have found this enzyme in liver (29), human erythrocytes (14) and in *Escherichia coli* (18, 20).

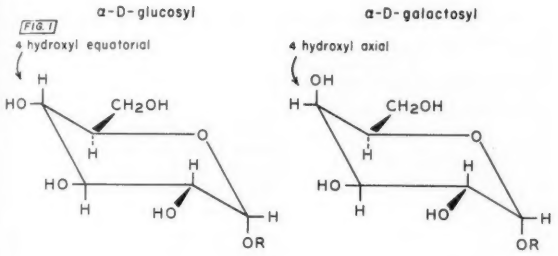
A few words about the connection between galactose and galactosides on one hand and glucose and its metabolites on the other hand. This relationship has been studied in the intact cell of the cow's udder with most ingenious methods by Harland G. Wood and his associates (40). The labeling pattern of lactose shall not be discussed here. However, these studies do give additional encouragement to the belief that the Leloir pathway plays the major role in the lactose-producing intact glandular cell.

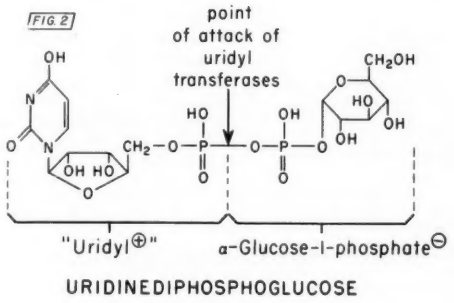
From the general scheme it seems clear that if external galactose is not supplied, formation of simple as well as complex galactosides from glucose metabolites requires an intact UDPGal 4-epimerase. Since Folch (7) has found that brain galactolipids are formed mainly after birth, at least in many mammalian species, it seems obvious that an intact functioning 4-epimerase is necessary for the formation of these lipids under conditions in which no external galactose is supplied. Assume that this applies likewise to the human species and keep this point in mind because it is not without importance in discussing the biochemical basis for the human hereditary disease, galactosemia.

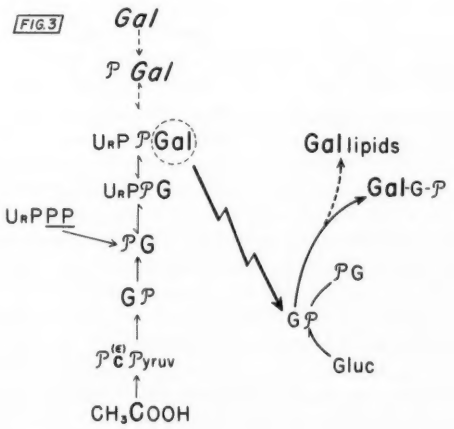
This hereditary disease has the following characteristics. It is a disease of the newborn. The disease becomes only manifest if the infant receives galactose. Levels of galactose in blood plasma are greatly increased. There is also excretion of galactose in the urine. Cellular abnormalities develop such as fatty degeneration in the liver, cataract and mental retardation or idiocy. Of considerable interest also for our biochemical argumentation is the fact that these infants can develop to healthy individuals if a strict galactose-free diet is instituted as early

[1] Contribution from the McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Md.

[2] Supported by grants from the National Institutes of Health and the National Science Foundation.







as possible. If instituted right after birth, disease may not develop at all and their mental capacity is normal or practically normal.

In 1955 a group of pediatricians in Manchester, England, discovered that administration of galactose to galactosemic infants brings about accumulation of galactose-1-phosphate in their red blood cells (32). The current opinion around this time was centered around 'galactowaldenase' (alias 4-epimerase) as the focus of disturbance. It is true that such a block in 4-epimerase would give rise to accumulation of Gal-1-P. (See later in the section on microbial galactose mutants.) However, as I stressed before, the interference of 4-epimerase combined with a strict galactose-free diet would almost inevitably involve the deposition of abnormal brain lipids such as glucolipids which may not be compatible with normal mental capacity. Personally I was, therefore, right from the beginning much more inclined to consider the disease either a single enzymatic block in Gal-1-P uridyl transferase or an involved disturbance in the regulation of galactose metabolism. In collaboration with Anderson and Isselbacher (12, 14), we went to work first on the red blood cells. The findings on the enzyme levels of the Leloir pathway in hemolysates of nongalactosemic and galactosemic individuals are summarized in table 1. The figures are average from about 20 cases of the first group and almost 40 cases of the second (13). As you can see, the galactokinase and the epimerase are not affected but the transferase is dramatically decreased. Kirkman (19) found that parents of galactosemic children have transferase levels which are moderately but definitely lower as compared with individuals from nongalactosemic families (see fig. 4 and table 1). Similar findings were reported by Brettauer, Hansen, Donnel and Bergren (2) and by Hsia *et al.* (11). The defect in transferase in galactosemics can also be seen in the red blood cells from umbilical cord blood (1) as well as in specimens from the liver (1). Later Lerman (27) found the same defect in lens epithelium and Weinberg (38) most recently demonstrated the defect in the white blood cells.

What I have so far told you seems at first glance to settle the main question concerning the biochemical basis for hereditary galactosemia. But at closer inspection our findings have mainly settled which enzyme is defective but not the nature of the defect. The latter, of course, may vary from family to family. You may have families which produce a protein which cross-reacts immunologically with normal transferase but has lost the catalytical activity. Or you may have cases in which no protein is found whatsoever. For a possible elucidation of these problems the recent findings by Topper and his group may be of considerable interest. They found that progesterone and especially menthol (6), administered to galactosemic individuals who also receive a small amount of radioactive galactose bring about as much production of radioactive carbon dioxide as found in a nongalactosemic subject. Without progesterone or menthol, galactosemics receiving radioactive galactose expire, at the most, only traces of radioactive carbon dioxide. What is the explanation of this interesting finding? Does men-

TABLE 1. *Quantitative Comparison of Activities of Enzymes of Galactose Metabolism in Three Classes of Subjects*

Classes of Subjects	Average Activities, μM Conversion/hr./g Cells (37°) (Lysates of Erythrocytes)			
	Galacto-kinase	Gal-1-P-uridyl transferase	UDP-Gal 4-epimerase	G-1-P, PP-uridyl transferase
Nongalactosemics	0.10	4.8	0.32	>10.0
Galactosemics	0.08	<0.02	0.35	>10.0
Galactosemic carriers		2.9		

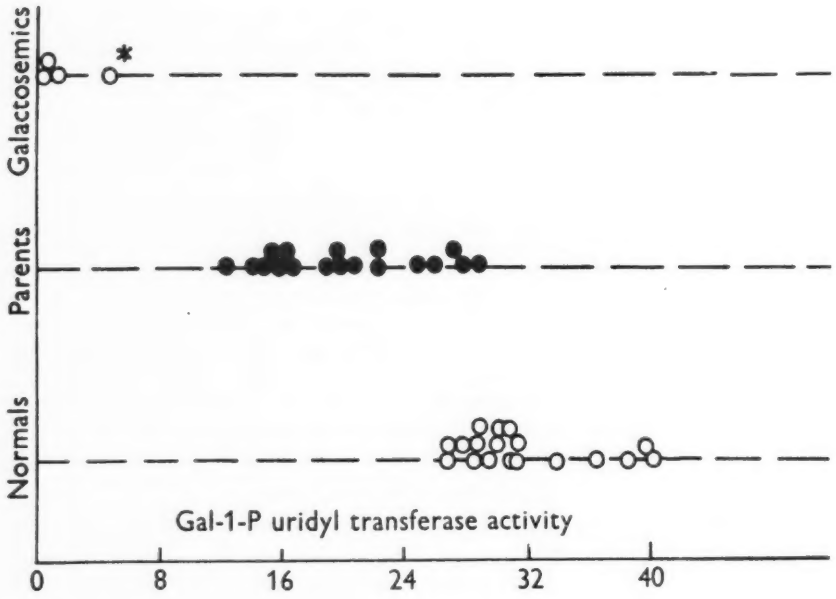


FIG. 4. Activity of Gal-1-P uridyl transferase expressed as μl. O_2 consumed per hr. by 0.3 g of red cells from three different classes of individuals. *a*) Non-galactosemics (mainly normal healthy individuals). *b*) Individuals suspected of being heterozygous with respect to the gene for the synthesis of the transferase since they have offspring with congenital galactosemia. *c*) Corresponding homozygous affected individuals, i.e. galactosemics. (Kirkman and Bynum, 1959a. Courtesy of *Ann. hum. Genet.*, Cambridge Univ. Press.)

thol stimulate the development of an alternative pathway which is shown to exist? Or might it even 'activate' the defective transferase? This question will have to await solution. Obviously we have much to learn about the biochemistry and physiology of hereditary galactosemia.

I shall now discuss some of our recent studies on bacterial mutants blocked in various steps of the Leloir pathway. In this discussion I shall limit myself largely to the so-called galactose-sensitive mutants. I shall first define a couple of notions: galactose-negative mutants are mutants which are unable to use galactose as a carbon source for growth and metabolism. Among those galactose negatives one can find galactose sensitives which are mutants that show signs of abnormal growth if galactose even in small amounts is added on top of another carbon source such as glycerol (13, 21). The mutants I shall discuss stem either from the Lederbergs or from Fukasawa and Nikaido in Tokyo. Most of the work to be discussed was performed jointly with Kurahashi, Yarmolinsky and Wiesmeyer.

In Lederbergs' *E. coli* K-12 mutants we have identified a number of strains with defects in one or more enzymes of the Leloir pathway (18, 20). For instance, corresponding to one group of mutants located in a particular cistron which E. Lederberg calls A (22), Kurashi found a number of galactokinaseless mutants (20). A group of other mutants located in cistron B (22) showed single defects in transferase (table 2). The latter kind turn out to be galactose sensitives. If galactose is added to a simple synthetic medium such as ammonia mineral medium with glycerol as a main carbon source, a striking interference with growth develops one to two generations after the addition of galactose (41). This is illustrated in figure 5. The ordinate represents growth in a logarithmic scale; the abscissa represents time. Note the rather abrupt cessation of growth 2 or 3 generations after galactose addition at a time when the synthesis of galactokinase has been well induced. Galactose-1-phosphate starts to accumulate, and late in stasis as much as 5 μmoles Gal-1-P per gram cells may accumulate (34). We call the state 'galactose-induced bacteriostasis' since we have proved that it is a true stasis and not a steady state level between dying and growing cells. The cells do not lyse at any time but viable counts show that the cells remain viable. The basis for the stasis as well for the prevention of lysis may be quite involved. Kurahashi has found that protein digests prevent the development of stasis (Kurahashi, unpublished observations). The effect of glucose as studied by Yarmolinsky, Wiesmeyer, Jordan and Kalckar (41) is particularly noteworthy. As you can see from figure 5 a galactose-induced stasis is broken within one-half hour after the addition of glucose. The rate of the renewed growth is somewhat lower than that of a culture in glycerol ammonia medium. The physiological explanation of the resumption of growth by the addition

TABLE 2. *Distribution of Enzymes Metabolizing Galactose in E. coli Mutants* (17)

Strain	Genetic notation	Galacto-kinase	Gal-1-P uridyl-trans.	UDP-Gal-4-epimerase	UDPG pyrophos-phorylase
W3100	Gal+	+	+	+	+
W3091	Gal_1−	+		+	+
W3092	Gal_2−		+	+	+
W3094	Gal_4−	+		+	+
W3096	Gal_6−	+		+	+
W3097	Gal_7−	+		+	+
W3178	Gal_8−		+	+	+
W3142	Gal−*		+	+	+

* No designation for this mutant, which is not linked in the same cluster as the others, has yet been published.

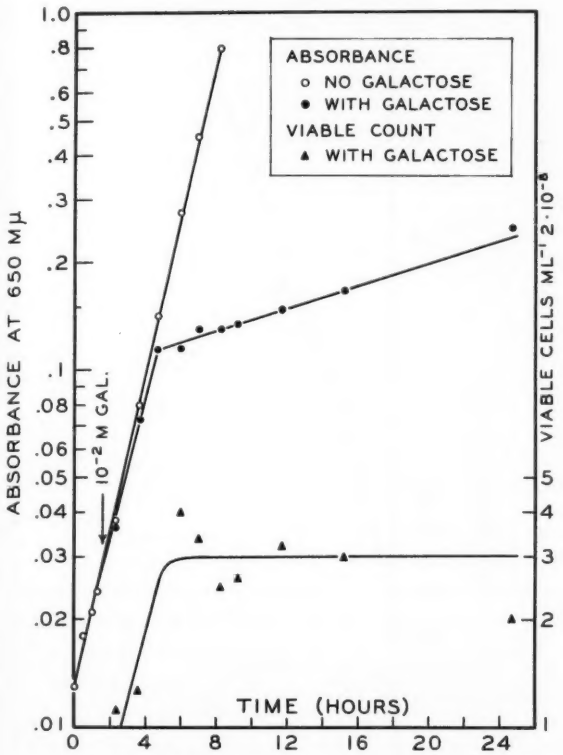


FIG. 5. Galactose-induced stasis of *E. coli* W 3104. Cells grown in shake culture at 37° in medium A containing 1% glycerol as carbon source. Galactose at a final concentration of 10^{-2} M added at the arrow.

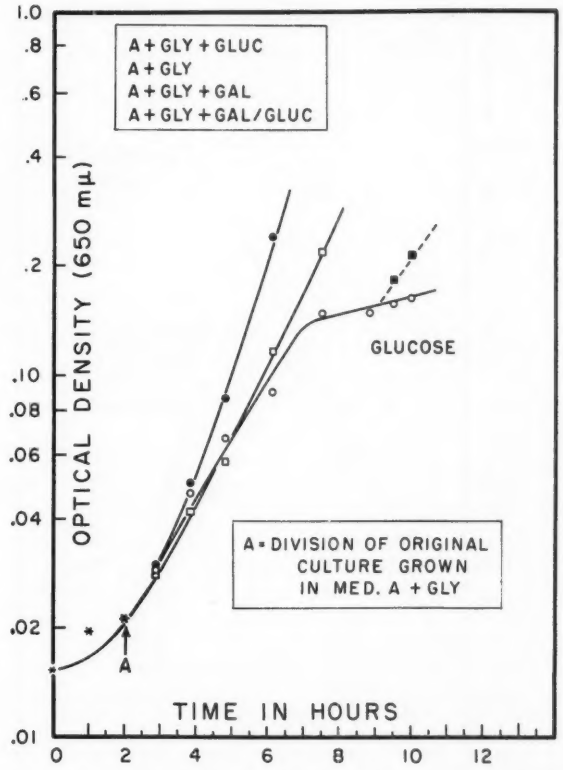

A + GLY + GLUC
A + GLY
A + GLY + GAL
A + GLY + GAL/GLUC
OPTICAL DENSITY (650 mμ)
GLUCOSE
A = DIVISION OF ORIGINAL CULTURE GROWN IN MED. A + GLY
TIME IN HOURS

FIG. 6. Effect of glucose on galactose induced stasis. A culture of W 3104 in medium A with glycerol as a carbon source was started (shake culture, 37°C). At point A the culture was divided into 3 parts. The first culture (●——●) received glucose (10^{-2} M); the second culture (□——□) was left unaltered; the third culture (○——○) received galactose (10^{-2} M). After 4 hr. additional growth the third culture showed stasis. After 2 to 3 hr. this culture was divided between two flasks. One flask was left unaltered; to the other flask (■——■) was added glucose (10^{-2} M). (Courtesy, *Proc. Natl. Acad. Sci. U. S.* [30].)

of glucose is not clear. It is, for instance, curious that the levels of Gal-1-P remain as high as 5 μmoles per gram even one and one-half generations after the renewed growth brought about by glucose (34). Perhaps some guidance may be found in the levels of UDPG (34). The UDPG-UDPGal levels which are 0.15 to 0.2 μmoles per gram cells during logarithmic growth drop below 0.05 μmole during stasis. Very soon after addition of glucose the UDPG levels 'climb' up to 0.2 μmole again. It is known that Gal-1-P inhibits phosphoglucomutase (10, 33) and it is not unlikely that this inhibition has something to do with the changes in the UDPG levels.

H. Friedmann working in our laboratory has most recently found another K-12 transferaseless mutant which manifests a different type of galactose sensitivity. If grown on glycerol ammonia medium, it lyses soon after the addition of galactose and here glucose does not have any effect.

About half a year ago Fukasawa and Nikaido in Tokyo were kind enough to send us some remarkable *E. coli* strains which they call C7M and which they found to lyse upon exposure to galactose even in a rich medium such as tryptone broth (8). In hypertonic sucrose lysis was prevented and spheroplasts or protoplasts were formed instead (8). We have conducted some biochemical studies on the C7M strains. Here is a brief summary of these studies which were performed jointly by Miss Jordan and Mr. Spyrides.

1) The enzyme defect in this strain is not in the transferase but in the epimerase (18) (table 3).

2) Galactose can act both as a bacteriostatic and as a bacteriolytic agent. In tryptone broth as little as 10^{-3} to 10^{-4}M of galactose causes bacteriolysis. In ammonia glycerol medium addition of galactose brings about bacteriostasis prior to bacteriolysis which follows inevitably (41).

3) Addition of glucose even at a late stage after the addition of galactose (i.e. at a time when the population shows signs of the onset of lysis) can salvage the population from lysis (41) (fig. 8).

4) Gal-1-P accumulates in amounts of 2 to 3 μmoles per gram cells (34).

5) There is a striking and characteristic accumulation of UDPGal (34). The absolute concentrations of UDPGal

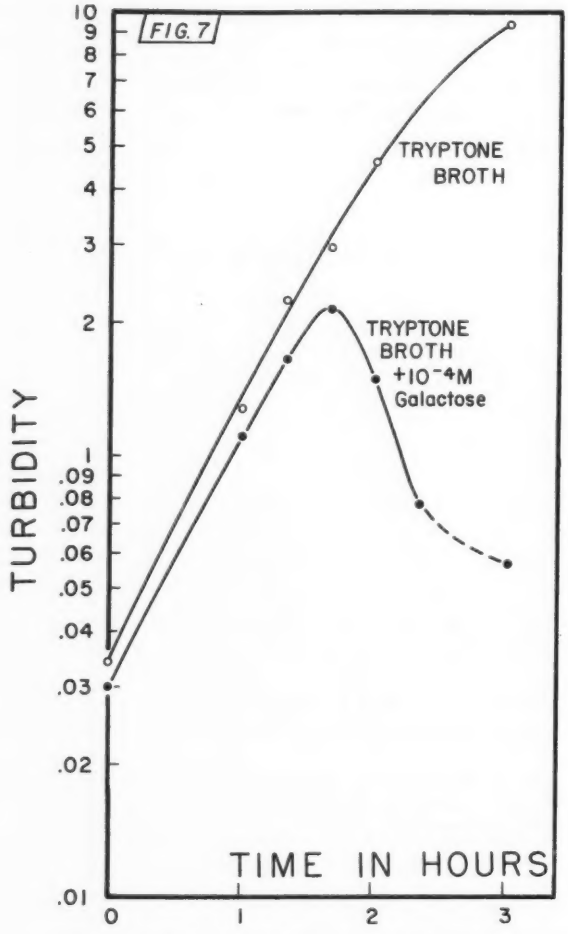


FIG. 7. Effect of addition of galactose to a culture of *E. coli* strain C7M, growing in tryptone broth (without glucose). The galactose used was D (+) galactose, C. P., Pfanstiehl. (Courtesy, *Proc. Natl. Acad. Sci. U.S.* [30].)

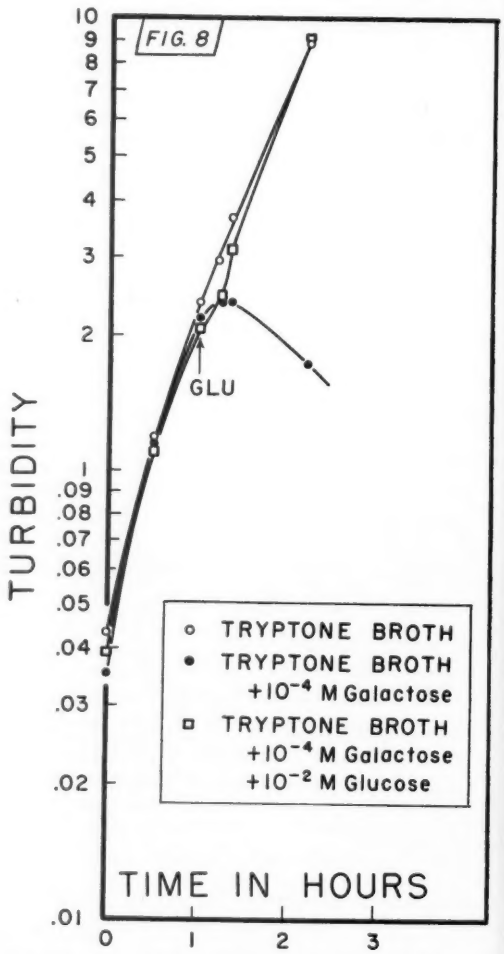


FIG. 8. Prevention of galactose-induced lysis of *E. coli*, C7M, by glucose. (Courtesy, *Proc. Natl. Acad. Sci. U.S.* [30].)

are greatly increased over the ordinary UDPG concentrations.

Let me elaborate on the latter point. UDP-glucose was determined by the specific enzyme UDP-glucose dehydrogenase described by Strominger, Maxwell, Axelrod and Kalckar (35). This highly specific dehydrogenase will not act on UDPGal. Upon addition of a highly fractionated liver 4-epimerase (28) as a second indicator enzyme UDPGal is converted to UDPG and now the dehydrogenase can act (cf 36). In cells in which the 4-epimerase is functioning normally we know already from Leloir's work (23) that we have 3 to 4 times as much UDP-glucose as UDP-galactose. The sum of both amounts to .1 to .2 μmole per gram cells in *E. coli* (34). In this epimeraseless mutant the ratio of UDPG to UDPGal in the presence of galactose in the growth medium is, as appears from figure 9, entirely altered. There are barely detectable amounts of UDP-glucose. In some cases there probably is no UDP-glucose left. In contrast the UDPGal concentration, which is normally below .05 μmole per gram wet weight, is not only relatively grossly increased but the absolute concentrations are strikingly higher than the sum of UDPG and UDPGal concentrations in cells grown in the absence of galactose. UDPGal concentrations as high as between 0.5 and 2.5 μmoles per gram cells are frequently observed (34). In other words, one can reach an increase of 50-fold over the concentration seen in cells grown in glycerol ammonia medium without galactose. It can also be seen from the same figure that this abnormal display of UDPGal remains just as pronounced after the addition of glucose which, as mentioned, causes the cells to grow again. The lack of UDPG seems, therefore, not to be the primary factor for the development of lysis.

The mutants which are blocked in galactokinase are not sensitive to galactose in the same way that the trans-

TABLE 3. *Phenotypic Description*

Genotype	Medium	Galactokinase, μM/mg protein/hr.		Gal-1-P Uridyltransf., μM/mg protein/hr.		4-epimerase, μM/mg protein/hr.	
		noninduc.	Gal-induc.	noninduc.	Gal-induc.	noninduc.	Gal-induc.
W3100*	A	<.05	40.0	0.6	5.5	5.0	40.0
W3100	Br†			0.4	5.8		
W3092	A	<.05	<0.05	5.2	4.3		42.0
W3078	56	<.05	<0.05	2.4	2.5		
W3142	56	<.05	<0.05	1.4	4.0		+
W3096	A	.10	15.0	<0.01	<0.01	2.0	23.0
W3096′	A	.20	2.1	<0.1	<0.06		6.4
W3104	A		+	<0.01	0.1 app.		+
W3099	A		0.05 app.		0.05 app.	<0.01	<0.01
W3264	A		0.10		0.35	0.60	0.90
W3265	A		0.10		0.30	0.50	0.80
ML32,400	A	<.1	<0.1		<0.1		+
C7M	Br		+	1.8	9.0	<0.01	<0.01

* The letter W is usually added to the numbers of the K-12 strains. † Br refers to tryptone broth.

ferase-less mutants are. Some of the galactokinase-less mutants synthesize transferase even in the absence of galactose in the medium and moreover to the extent of full constitutivity. Other kinase-less mutants synthesize transferase like the wild type (which is galactose positive), i.e., on an inducible basis (Yarmolinsky and Jordan, unpublished observations). There is no evidence that Gal-1-P is a precursor of repressors for transferase synthesis. The idea that galactokinase may catalyze the formation of repressors for transferase synthesis (for instance, by phosphorylation of sugars occurring in the K-12 cells) should, however, be seriously considered. A major defect in galactokinase would bar phosphorylation of galactose as well as formation of repressor of transferase synthesis. However, in the kinase-less mutants which have retained the repression of transferase synthesis one might assume that the catalysis of repressor formation is preserved whereas the catalysis of galactose phosphorylation has been lost. Of course, the interpretation can also be turned around, assuming that the state in which transferase synthesis is constitutive is due to a highly developed ability to inactivate repressors for transferase (17). The possibilities for the interpretation of the interesting correlations are undoubtedly not exhausted with the above-mentioned hypothetical mechanisms.

In our laboratory Wiesmeyer and Yarmolinsky (39) have made interesting studies on transferase synthesis as influenced by the presence of the bacterial virus, lambda (λ), and the 'galactose-transducing lambda', 'λ-gal'.

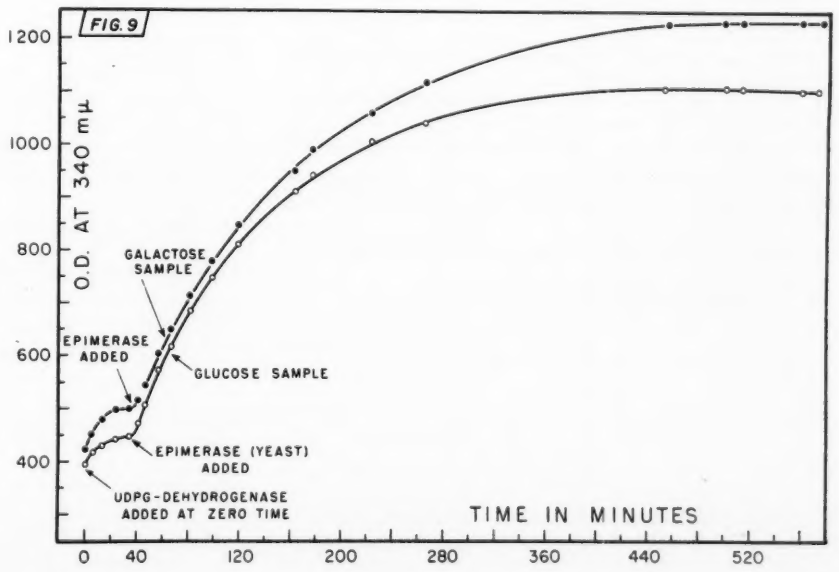


FIG. 9. Illustration of UDPG/UDPGal ratios in *E. coli*, C7M by stepwise enzymic spectrophotometry. UDPG is determined by addition of UDPG dehydrogenase, UDPGal by addition of yeast UDPGal 4-epimerase. Abscissae: time in minutes after addition of the indicator enzyme. Ordinate: formation of reduced DPN brought about by dehydrogenase or the combination of this enzyme with epimerase. 'Galactose sample' (●——●) refers to the protein free filtrate from galactose grown cells. 'Glucose sample' (○——○) refers to the filtrate from cells grown in galactose but with the further addition of glucose.

When an *E. coli* strain lysogenic for either λ or λ and λ-gal is exposed to ultraviolet irradiation to induce phage development, a conversion from provirus to vegetatively multiplying phage takes place. Towards the end of the latent period, at a time when the viral DNA seems to have undergone replication, a sudden increase in the specific activity of transferase occurs in each case (39). The increase is 15 to 30-fold on a cellular level and 7 to 15-fold based on specific activity, the lower values applying to strains lysogenic for λ alone. Transferase is obviously unessential for the vegetative cycle of λ and λ-gal and not a constituent of the viruses. Hence, this extensive synthesis is entirely gratuitous.

It should be mentioned that a group at the Institut Pasteur (4) has studied the status of galactokinase synthesis under similar conditions with similar results. Their arguments concerning problems of repression and derepression are somewhat beyond the scope of this discussion.

These microbiological studies have encouraged us to initiate metabolic studies on tissue cultures from galactosemic subjects, galactosemic carriers (heterozygotes) and nongalactosemic subjects. It is my belief that galactosemia offers a promising field for studying the action of genes in man, since it is in some respects a fairly well-defined biochemical disease which affects many types of mammalian cells. Yet let me not fail to emphasize the need for more knowledge concerning the property of the defect. One of the approaches first to be put into action would obviously be the immunological technique, but many other approaches should be pursued.

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Genetic and biochemical lesions of polysaccharide synthesis in the pneumococcus[1]

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THE DEMONSTRATION by Avery, MacLeod and McCarty in 1944 (5) of the ability of highly polymerized DNA preparations from a capsulated Type II pneumococcus to induce the formation of capsulated Type III cells when added to an actively growing culture of noncapsulated Type II pneumococci opened a new chapter in the study of the transfer of genetic characters.

That the active transforming principle is DNA has been amply demonstrated by the more recent studies of McCarty and Avery (20), Hotchkiss (14) and Zamenhof (31, 32). Other factors, such as drug resistance (15), the ability to modify enzymic activity (4, 9, 19) have also been shown to be hereditarily determined by DNA in the pneumococcus. The transfer of genetic characters by isolated DNA has also been demonstrated in other bacterial species (30) and is now a well established phenomenon.

In the pneumococcus, over 70 different capsular types are known, their differentiation being determined by immunologically distinct capsular polysaccharides. The present discussion will, however, be restricted to two capsular types and an attempt will be made to correlate information on the enzymic steps involved in capsular polysaccharide synthesis with data obtained from genetic studies. The two types chosen for study here are Types I and III. The exact nature of the Type I capsular polysaccharide is at present unknown, but the components so far shown to be present are galacturonic acid (60%), an amino sugar (8%), fucose and an unidentified monosaccharide (E. E. B. Smith, B. Galloway and G. T. Mills unpublished observations). Type III capsular polysaccharide has the well defined structure (1, 16, 22):

$$-(3\text{-}\beta\text{-D-glucuronic acid-1} \rightarrow 4\text{-}\beta\text{-D-glucose-1} \rightarrow)_n$$

The similarity in the two polysaccharides is the presence in each of a large amount of a uronic acid. The pathway of formation of the uronic acid component of each polysaccharide will be of obvious importance for the synthesis of the respective capsular materials.

It was found that capsulated Type III pneumococci contain UDPglucose (UDPG), UDPgalactose (UDPGal) and UDPglucuronic acid (UDPGA) (28). From capsulated Type I pneumococcus, the nucleotide UDPgalacturonic acid (UDPGalA) has also been isolated (29). Originally UDPglucuronic acid had not been found in Type I organisms but more recent work has shown that the failure to detect this nucleotide is a function of the growth phase of this organism and that in its resting phase both UDPGA and UDPGalA can be found. The mechanism of formation of these nucleotides in both strains of pneumococcus has been studied in cell free extracts obtained by disintegration and ammonium sulphate fractionation (23–25, 28).

It has been found that the mechanism of formation of glucuronic acid in pneumococcus is very similar to that found in mammalian liver (21). In the fully capsulated strains of Type I and Type III pneumococcus the following enzymes have been shown to be present.

1) *Uridyl transferase*, responsible for the reaction:

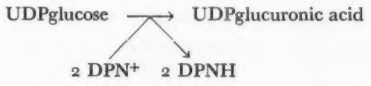
$$\text{UTP} + \text{G-1-P} \rightleftharpoons \text{UDPG} + \text{PP}_i$$

2) *UDPGal-4-epimerase*, responsible for the reaction:

$$\text{UDPG} \rightleftharpoons \text{UDPGal}$$

The properties of these two enzymes are similar to those found in yeast and mammalian tissues. In addition to being present in the fully capsulated cells both enzymes are present in all noncapsulated mutants of Type I and Type III pneumococcus which have been studied.

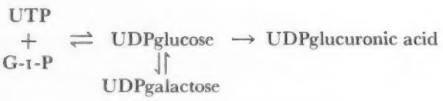
3) *UDPglucose dehydrogenase:*



This enzyme shows optimal activity at pH 8.4, requires DPN, is activated by Mg^{++} and would appear to be an—SH enzyme since it is readily inactivated by H_2O_2 and protected from such inactivation by thioglycolate.

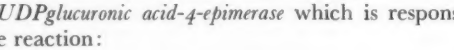
These reactions establish the following pathway in both Types I and III.

[1] The experimental work reported here is the result of a very close collaboration between the author and his wife (Dr. Evelyn E. B. Smith) and with Drs. Robert Austrian and Harriet P. Bernheimer, the latter two workers having carried out the genetic studies. This work was supported in part by a grant (E-1018(C4)) from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.



In most pneumococcal types studied, a conventional pathway of glycolysis appears to be present (26, 28). In the case of capsulated Type I pneumococci an additional enzyme has been demonstrated; namely,

4) UDPglucuronic acid-4-epimerase which is responsible for the reaction:



This enzyme probably requires DPN since, although it is active in the absence of added DPN, the addition of DPN stimulates the reaction and DPNase abolishes activity (23, 25).

While the uronic acids are synthesized in the pneumococcus by the uridine nucleotide pathway, it remains necessary to show that the UDPglycosyl compounds act as the immediate donors of the glycosyl units for capsular polysaccharide synthesis. The role of UDPglycosyl compounds in the synthesis of some polysaccharides is already well documented (table 1). It would appear possible from this already published work that pathways involving UDPglycosyl compounds may represent a general mechanism for polysaccharide synthesis.

In view of the simple nature of the polysaccharide produced by pneumococcus Type III, this organism was chosen to study the role of UDPG and UDPGA in polysaccharide formation. Enzyme extracts from Type III organisms contain large amounts of capsular polysaccharide; and, to reduce any possible interference by this material, it was largely removed by growing the organisms in the presence of the specific Type III capsular polysaccharide depolymerase of Dubos (8). This enzyme removes the capsule of Type III pneumococcus without interfering in any way with the ability of the cells to synthesize more polysaccharide (7). The enzyme extracts prepared from such cells contained only small amounts of Type III capsular polysaccharide and were tested for their ability to incorporate C^{14} glucose and C^{14} glucuronic acid from the UDP compound into Type III polysaccharide (table 2) (27). All the radioactivity of the isolated polysaccharide is specifically precipitable with Type III pneumococcal capsular antiserum and quantitatively recoverable by dissociation of the antigen-antibody complex. The radioactivity is rendered dialyzable when the polysaccharide is hydrolyzed with the specific Type III capsular polysaccharide depolymerase.

TABLE 1 *Synthesis of Polysaccharides from UDPglycosyl Compounds*

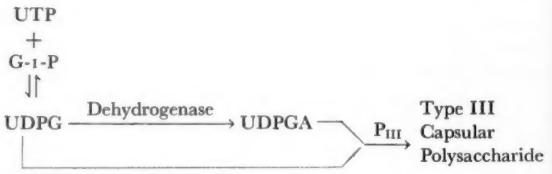
UDP Compound	Polysaccharide Synthesized
UDPAG	Chitin (13)
UDPG	Cellulose (12)
UDPG	Glycogen (17)
UDPG	β-1-3-glucan (11)
UDPGA and UDPAG	Hyaluronic acid (18)

The site of labeling of the polysaccharide was found to be entirely dependent upon the labeled substrate used (table 3). There was found to be no incorporation of isotope when C^{14} glucose or C^{14}-glucose-1-phosphate were substituted for C^{14} UDPG.

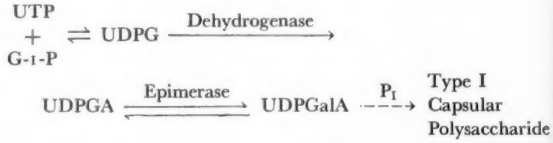
These experiments provide good evidence for the incorporation of glucose and glucuronic acid from their respective UDP compounds into the Type III capsular polysaccharide. To date no similar work has been done with Type I capsular polysaccharide.

The over-all pathway of uronic acid synthesis in the capsulated Type III and Type I cells may be pictured as follows.

Type III:



Type I:



An examination has been made of the biochemical capabilities of a number of noncapsulated mutants of Type I and Type III. They produce little or no capsular

TABLE 2. *Incorporation of C^{14} Glucose and C^{14} Glucuronic Acid from UDPG and UDPGA into Type III Pneumococcal Capsular Polysaccharide*

Radioactivity of substrates = 150 × 10^3 c.p.m./μmole

Substrate amount	Incubation time min.	Type III Capsular Polysaccharide Isolated	
		amt. μg	Specific activity c.p.m./mg
2 μmoles C^{14} UDPG + 1 μmole UDPGA	0	77	0
2 μmoles C^{14} UDPG + 1 μmole UDPGA	30	95	74,300
2 μmoles UDPG + 1 μmole C^{14} UDPGA	30	113	62,300
2 μmoles C^{14} UDPG + 1 μmole C^{14} UDPGA	30	123	120,000

TABLE 3.

Labeled Substrate	Labeling in Type III Polysaccharide
UDPG	Glucose
UDPGA	Glucuronic acid
UDPG + UDPGA	Glucose + glucuronic acid

TABLE 4. *Type III Cells*

	Capsule	UDPGA	UDPG Dehydrogenase
SIII	+	+	+
$S\text{-}III_1$	−	− to trace	−
$S\text{-}III_2$	−	− to trace	−
$S\text{-}III_3$	−	− to trace	−
$S\text{-}III_4$	−	− to trace	−

TABLE 5. *Type I Cells*

	Capsule	Nucleotides		Enzymes
		UDP-GA	UDP-GalA	
SI	+	+	+	complete
$S\text{-}I_1$	−	+	−	UDPG dehydrogenase + UDPGA epimerase −
$S\text{-}I_2$	−	−	−	UDPG dehydrogenase − UDPGA epimerase +
$S\text{-}I_3$	−	+	−	UDPG dehydrogenase + UDPGA epimerase −

polysaccharide, and they have been examined for their ability to produce UDPGA and for the presence of UDPG dehydrogenase in the case of Type III mutants and for the ability to produce UDPGA and UDPGalA and the presence of UDPG dehydrogenase and UDPGA-4-epimerase in the case of Type I mutants. The results of such examinations are summarized in tables 4 and 5 (2, 23).

The significant finding of Austrian and Bernheimer (3) that, when a noncapsulated Type III strain ($S\text{-}III$) was transformed with DNA from a fully capsulated Type I strain, there appeared a small proportion of a new type of cells exhibiting the presence of two capsular polysaccharides (I and III) in addition to the expected Type I cells, opened a new phase in the understanding of the mechanism of capsular transformation in the pneumococcus.

$$S\text{-}III\ (\text{UDPG dehydrogenase} -) \xrightarrow[SI]{DNA} \begin{matrix} SI \\ SI\text{-}III \end{matrix}$$

In this transformation the number of Type I cells recovered is greater than the number of binary capsulated Type I-III cells. It has been found that the amount of Type III capsulated polysaccharide on the SI-III approximates that normally found on a wild Type III cell, whereas the amount of Type I polysaccharide is less than that of the Type I transformants.

When DNA from the binary capsulated SI-III cells is used to transform noncapsulated cells of a heterologous type (namely Type II), segregation of the genetic factors concerned with capsular synthesis may take place.

$$S\text{-}II \xrightarrow[SI\text{-}III]{DNA} \begin{matrix} SI \\ S\text{-}III \\ SI\text{-}III \end{matrix}$$

TABLE 6. *Incorporation of C^{14} Glucose from UDPG in the Presence of UDPGA into Type III Pneumococcal Capsular Polysaccharide by Extracts of a noncapsulated Type III Pneumococcus*

Radioactivity of UDPG = 150×10^3 c.p.m./μmole

Substrates	Incubation time min.	Type III Capsular Polysaccharide Isolated	
		Amount μg	Specific activity c.p.m./mg
1 μmole C^{14} UDPG + 1 μmole UDPGA	30	66	203,000

No fully capsulated SIII cells have ever been recovered in transformations of this kind, indicating that the $S\text{-}III$ genome has not been restored to normal in the binary capsulated cells. It is probable, therefore, that in the binary capsulated SI-III cells, there has been no genetic exchange between the Type I capsular genome and the mutated Type III capsular genome. The restoration of Type III capsular polysaccharide production appears to take place at the metabolic level, the Type III synthetic pathway utilizing UDPglucuronic acid from the Type I pathway.

$S\text{-}III$ (UDPG dehydrogenase −):

UDPG ⟶// UDPGA → Type III capsular polysaccharide

SI: UDPG → UDPGA → UDPGalA → Type I capsular polysaccharide

In this case, there is competition between two pathways for the available UDPGA. This fact would explain the production of subnormal amounts of Type I polysaccharide with augmented amounts of Type III polysaccharide by the SI-III cells.

Another example of this mechanism of interaction is seen when the noncapsulated Type III cell $S\text{-}III$ is transformed with DNA from the noncapsulated Type I cell ($S\text{-}I_1$ (UDPGA epimerase −, UDPG dehydrogenase +)). In this case, only capsulated SIII phenotypes arise.

$$S\text{-}III(\text{UDPG dehydrogenase} -) \xrightarrow[S\text{-}I_1\ (\text{UDPGA epimerase} -)]{DNA} SIII(I)^2$$

$S\text{-}III$:UDPG ⟶// UDPGA → Type III capsular polysaccharide

$S\text{-}I_1$ UDPG → UDPGA ⟶// UDPGalA----

Here again the production of UDPGA by the metabolic

[2] Parentheses indicate mutated Type I capsular genome carried cryptically.

TABLE 7. *UDPG Dehydrogenase and UDPGA-4-Epimerase Activities of Several Strains and Variants of Type I Pneumococcus*

Type I Strain	Relative UDPG Dehydrogenase Activity	UDPG-A Epimerase
SI	100	+
$S\text{-}I_1$	34	−
$S\text{-}I_2$	0.9	+
$S\text{-}I_3$	107	−
$S\text{-}I_1 \xrightarrow[S\text{-}I_2]{DNA} SI$	35	+
$S\text{-}I_3$ Spontaneous reversion → SI	60	+

pathway determined by mutated Type I capsular genome bypasses the block in the altered synthetic pathway of the noncapsulated mutant of pneumococcus Type III. These experiments indicate that the metabolic block in the $S\text{-}_{III}$ mutants studied involves only UDPG dehydrogenase and that the polymerizing system is fully active. This conclusion was tested experimentally by studying the incorporation of isotope into Type III capsular polysaccharide from C^{14} labeled UDPG in the presence of UDPGA when using an enzyme preparation from an $S\text{-}_{III}$ strain which produces only a minute amount of Type III capsular polysaccharide. These cells were also treated with capsular depolymerase during growth and the experiment carried out as already described. The result in table 6 indicates a rate of polysaccharide formation of the same order as that of fully capsulated Type III cells.

Interactions at the metabolic level which occur following transformation are different from the allogenic transformations in the $S\text{-}_{III}$ series described by Ephrussi-Taylor (10). In this case the transformation of $S\text{-}_{III}$ cells with DNA from a different $S\text{-}_{III}$ strain gives rise to Type III cells producing considerable amounts of capsular polysaccharide.

$S\text{-}III_4$ (UDPG dehydrogenase −)

$$\xrightarrow[S\text{-}III_2(\text{UDPG dehydrogenase} -)]{\text{DNA}} \text{SIII}$$

DNA from the SIII cells recovered behaves as that from wild Type III cells, this observation indicating that there has been interaction at the genetic level. The genetic lesion in all these $S\text{-}_{III}$ strains appears to involve locus controlling UDPG dehydrogenase and it seems likely that these are nonidentical mutations affecting the same cistron (6).

In the case of the noncapsulated mutants of Type I pneumococcus it also appears that there is only a single block in the polysaccharide synthesizing pathway in each of the strains studied. On transformation of $S\text{-}_I$ (UDPGA epimerase —) cells with DNA from $S\text{-}_I$ (UDPG dehydrogenase −) cells, fully capsulated SI cells are recovered.

Complete Type I pathway:

SI: UDPG → UDPGA → UDPGalA → Type I capsular polysaccharide

Mutated Type I pathways:

$S\text{-}I_1$: UDPG → UDPGA ⫽⫽ UDPGalA ----

$S\text{-}I_2$: UDPG ⫽⫽ UDPGA → UDPGalA ----

Result of transformation:

$$S\text{-}I_1\begin{pmatrix}\text{UDPGA epimerase} -\\ \text{UDPG dehydrogenase} +\end{pmatrix}$$

$$\xrightarrow{\text{DNA}} \text{SI}$$

$$S\text{-}I_2\begin{pmatrix}\text{UDPG dehydrogenase} -\\ \text{UDPGA epimerase} +\end{pmatrix}$$

The restitution of a normal Type I capsular genome by transformation leads to a restitution of a normal metabolic pathway.

In table 7 are listed the relative UDPG dehydrogenase activities of a number of Type I variants.

The restitution of polysaccharide synthesis to normal after transformation takes place as a result of genetic recombination, since the capsular genome of Type I cells recovered from this transformation behaves as that of wild type cells in all future transformations. This recombination involves two "cistrons," thus distinguishing it from that in the $S\text{-}_{III}$ series where one cistron is involved.

From these metabolic and genetic experiments, one may conclude that the various units comprising the capsular genome are closely linked since, in the case of the Type I capsular genome, the control of at least three and probably more biochemical functions is determined by it. Following transformation the frequency of appearance of SI cells is too high to be accounted for if the units are unlinked.

In the binary capsulated SI-III cells, while there may be some 'linkage' between the SI and the $S\text{-}_{III}$ genomes in view of the ability of DNA from SI-III cells to transform unrelated noncapsulated types to SI-III cells, this 'linkage' is probably of a very low order. It differs from that linking the several genetic determinants concerned with the synthesis of Type I or Type III capsular polysaccharide, for segregation of these determinants has not been observed.

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Glucose-6-phosphate dehydrogenase deficiency in hemolytic anemia[1]

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Unforeseen results having significance in several biological areas have arisen from investigations of antimalarial drugs undertaken by Alving and associates at the Army Medical Research Unit of the University of Chicago in 1944. Initially, the 8-aminoquinoline, primaquine, 8-(4-amino-1-methylbutylamino)6-methoxyquinoline, was found to precipitate an acute intravascular hemolysis in approximately 10% of Negro American males when administered in twice the therapeutic dose necessary for radical cure of vivax malaria (53). The Chicago group of investigators began a study of the mechanism of hemolysis in 1951 with the hope that its elucidation might lead to the development of safer therapeutic agents. The intent of this paper is to present a perspective of the rapidly expanding field which has derived from this study.[2]

DEVELOPMENT AND PRINCIPAL FINDINGS

By 1955 Dern, Beutler, Alving and their associates had been able to report that the hemolytic defect was intrinsic in the red blood cell (34); that the hemolysis was self-limited (32), a function of cell age (12); and that hemolytic susceptibility was a manifestation of multiple drug sensitivity (33). The same group showed, by morphologic and immunologic studies, that the cells could be distinguished from normal only by an increased tendency to develop toxic granules, Heinz bodies, visible microscopically (11, 13).

The typical course of acute intravascular hemolysis

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[2] A recent review of the hemolytic effect of primaquine has appeared (10). Some of other reviews and symposia pertinent to the material to be presented are listed in the references (1, 6, 25, 29, 31, 54, 68, 69, 71, 75, 85, 87, 117, 119).

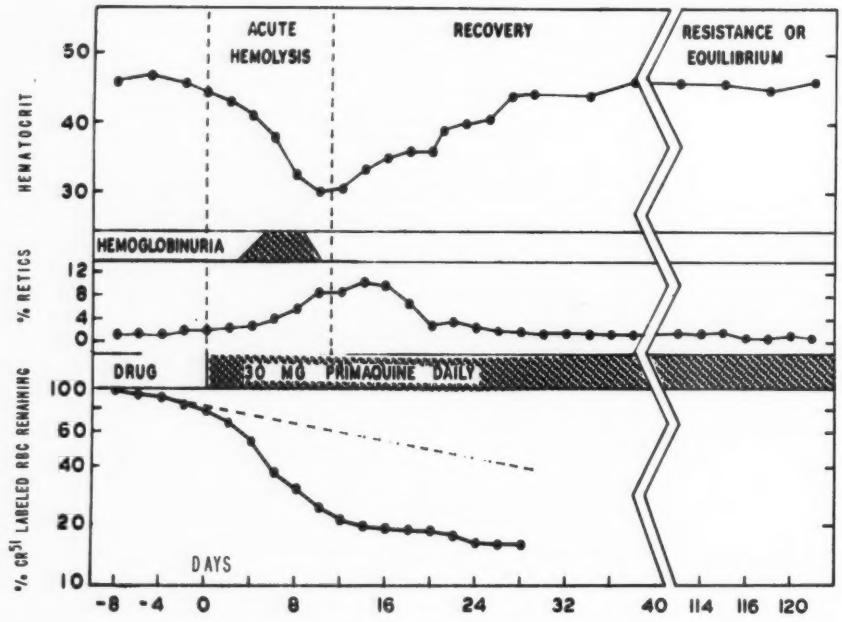

FIG. 1. Typical course of hemolysis induced in hemizygous males by daily administration of 30 mg primaquine base. (Reprinted from Bull. WHO 22, 625. 1960.)

which takes place in sensitive males on administration of 30 mg of primaquine base daily is shown in figure 1. Hemolysis begins within 3 or 4 days and continues for about 1 week, when recovery ensues even if administration of the drug is continued at the same dose. The resistance to further hemolysis is relative, however. If the dose is suddenly greatly increased, for example to 120 mg per day, a second hemolysis will occur (61). Administration of as much as 240 mg/day of primaquine base will cause little if any hemolysis in a nonsensitive individual.

The first biochemical abnormality of primaquine-sensitive erythrocytes, a low content of reduced glutathione, was described by Beutler *et al.* (14) early in 1955. In addition, Flanagan *et al.* (42, 43) reported that the reduced glutathione level fell abruptly at the initiation of an hemolysis and appeared to begin this fall before other major evidence of hemolysis became apparent.

These alterations of erythrocytic glutathione stimulated our interest in glutathione reductase which, though first demonstrated as a specific enzyme in plants (26, 73), is apparently ubiquitous throughout the biological world (29).

The reaction is:[3]

(1) $$GSSG + TPNH + H^+ \xrightarrow[\text{REDUCTASE}]{\text{GSSG}} 2\ GSH + TPN$$

The preferred coenzyme is TPNH (25).

For practical purposes, the reaction proceeds stoichiometrically to completion *in vitro*. There is apparently no metal requirement and optimum activity may be ensured by addition of EDTA. Reduced coenzyme for the reaction may be provided either directly or by coupling (25) with reactions which will provide TPNH such as the following:

(2) $$G\text{-}6\text{-}P + TPN^+ \xrightleftharpoons{\text{G-6-PD}} 6\text{-}P\text{-}G + TPNH^+ + H^+$$

(3) $$6\text{-}P\text{-}G + TPN^+ \xrightleftharpoons{\text{6-P-GD}} \text{Pentose-phosphate} + CO_2 + TPNH + H^+$$

Coincidentally, at the time of these studies, these reactions also represented the two successive oxidative steps of the pentosephosphate pathway. In figure 2 are shown the results obtained when dialyzed hemolyzate was used as source of the enzymes. Glutathione reductase and 6-phosphogluconic dehydrogenase were present in the hemolyzates from both primaquine-sensitive and nonsensitive cells. However, G-6-PD in hemolyzates of primaquine-sensitive erythrocytes was deficient as indicated by its failure to couple with glutathione reductase (19) (shown in the middle section of the figure).

The excitement of this discovery quickly shifted to the questions raised rather than those answered. Certainly, the abnormality of the oxidative pathway of carbohydrate metabolism was linked with the altered content

[3] Abbreviations used are as follows: GSH, reduced glutathione; GSSG, oxidized glutathione; TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; 6-P-G, 6-phosphogluconate; 6-P-GD, 6-phosphogluconic dehydrogenase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; EDTA, ethylene-diaminetetraacetic acid; DEAE, diethylaminoethanol cellulose.

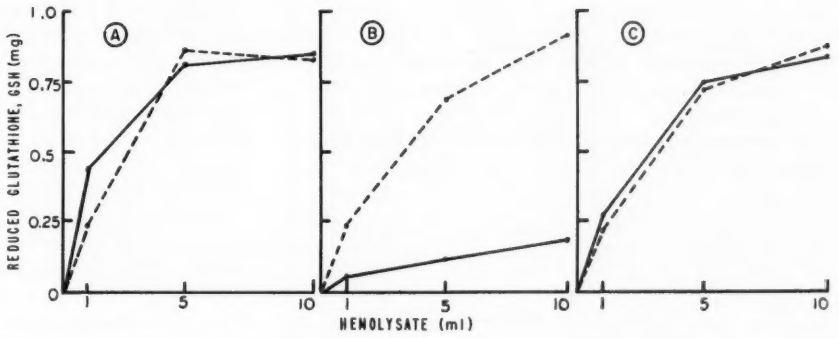

FIG. 2. Milligrams of reduced glutathione formed from oxidized glutathione as a function of milliliters of dialyzed hemolyzate. Solid curve, primaquine-sensitive subject; dashed curve, normal subject. *A*) Assay of GSSG reductase, reaction 1; *B*) assay of G-6-P dehydrogenase, reaction 2 + 1; *C*) assay of 6-P-G dehydrogenase, reaction 3 + 1. (Reprinted from Science 124: 484. 1956.)

and stability of glutathione. This did not suffice to explain the mechanism of hemolysis but the importance of the pentosephosphate pathway in relation to glutathione metabolism and maintenance of erythrocytic integrity was heightened by the finding of Schrier *et al.* (104) of a greater activity of glutathione reductase in primaquine-sensitive cells than in nonsensitive cells.

Probably the most significant effect of this discovery has been heuristic, with the deficiency of G-6-PD serving as the integrating factor. The problems posed can be classified on several levels.

1) Molecular. Was the enzyme only quantitatively deficient or could qualitative changes be demonstrated? What would be the role of activating or inactivating factors which might be present? Was this enzymatic deficiency primary or would it, in turn, prove to be secondary to still another altered molecular constituent of the primaquine-sensitive red cell?

2) Cellular. What was the mechanism of hemolysis? How were the metabolic pathways further altered by the hemolytic products of ingested agents to cause lysis of the cell? Would this prove to be qualitatively or quantitatively different from the cellular destruction of red cells known normally to take place in 120 days, i.e., what was the relation to cell age? Why was there no clinical hemolysis unless an hemolytic agent was ingested? How would this drug-induced hemolysis differ from that induced by other means, for example, immunologic, either *in vivo* or *in vitro*? What means, if any, could be found to protect against such an hemolysis? Would cells other than erythrocytes also exhibit a deficiency of glucose-6-phosphate dehydrogenase or any of the related biochemical changes found in the nonnucleated erythrocytes?

3) Whole organism. How would these biochemical changes be related to the overall metabolism of the whole body under either physiological or pathological conditions? What was the genetic basis for expression of the primaquine-sensitive pattern of biochemical abnormalities?

Subsequent to these initial investigations, contributions to the study of this entity in individuals whose erythrocytes have the primaquine-sensitive pattern have appeared from many laboratories. The list of hemolytic agents continues to expand and includes several drugs which are in common clinical use; for example, various sulfonamides (10, 33, 60), nitrofurans (62) and vitamin K derivatives (123). Naphthalene (moth balls) will cause hemolysis (122). British antilewisite (dimercaprol) (Tarlov, personal communication) and methylene blue (Brewer, personal communication) have recently been found to cause hemolysis in primaquine-sensitive individuals.

A most fascinating facet of primaquine sensitivity was unearthed when it was found in Italy, by Sansone and Segni (101, 102), and Larizza and associates (68, 69); in Israel, by Szeinberg, working with Sheba and associates (111, 112), and in the United States by Zinkham, *et al.* (125), that favism, an acute hemolytic anemia appearing in some individuals after eating fava beans, a staple in the Mediterranean diet, occurred only in people who had the primaquine-sensitive pattern in their red cells. The entity has now been found in all races (10, 16, 116, 118), though often occurring in particular regions or groups. For example, in the Italian region it is found primarily in Sardinians, or mainlanders of Sardinian descent (Brunetti, personal communication). In Israel, the disorder occurs in Sephardic or Oriental Jews, but not in Ashkenazy or European Jews (112). The incidence varies from 1–2% to as much as 30–40% among certain affected peoples (10). We can estimate that there are probably more than 100,000,000 people who have the primaquine-sensitive pattern of biochemical abnormalities in their erythrocytes.

Childs *et al.* (24) who demonstrated that primaquine-sensitivity is genetically determined, utilized one of the parameters of primaquine hemolysis, namely, the *in vitro* instability of glutathione demonstrated by Beutler (9). Other genetic studies have also included determinations of glucose-6-phosphate dehydrogenase (G-6-PD) activity (10, 23). Although the inheritance is not fully worked out, the presently most useful concept is that transmission is by a sex-linked gene of intermediate dominance (23). The result is that males and homozygous females have full expression of the trait, but heterozygous females show wide variation in their expression and also undergo intermediate hemolysis.

During the development of the information thus far presented, the activities of most of the enzymes shown in figure 3 were tested and two, glutathione reductase (104)

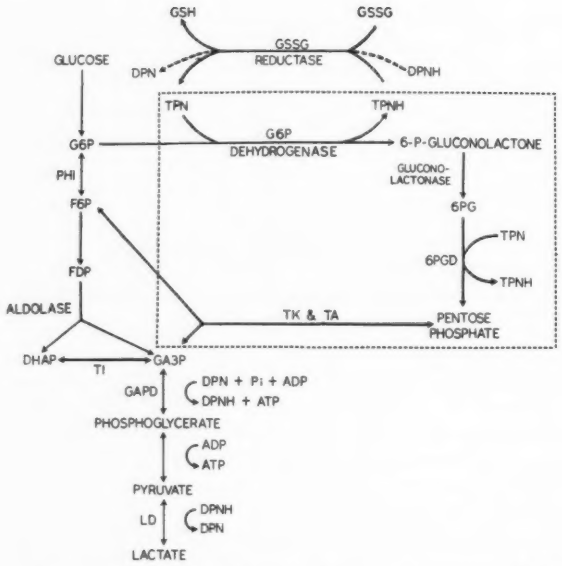

FIG. 3. Glucose catabolism in human erythrocytes. *GSH:* reduced glutathione; *GSSG:* oxidized glutathione; *G-6-P:* glucose-6-phosphate; *PHI:* phosphohexoseisomerase; *F-6-P:* fructose-6-phosphate; *FDP:* fructose 1-6-diphosphate; *DHAP:* dihydroxacetone phosphate; *GA3P:* glyceraldehyde-3-phosphate; *TI:* triose isomerase; *GAPD:* glyceraldehyde-3-phosphate dehydrogenase; *LD:* lactic dehydrogenase; *TPN:* oxidized triphosphopyridine nucleotide (coenzyme II); *TPNH:* reduced triphosphopyridine nucleotide; *DPN:* oxidized diphosphopyridine nucleotide (coenzyme I); *DPNH:* reduced diphosphopyridine nucleotide; *6-PG:* 6-phosphogluconate; *6PGD:* 6-phosphogluconic dehydrogenase; *TK:* translcetolase; *TA:* transaldolase; *Pi:* inorganic phosphate; *ADP:* adenosine diphosphate; *ATP:* adenosine triphosphate.

and aldolase (105), were found to be elevated. 6-Phosphogluconic dehydrogenase (19, 56, 104), transketolase and transaldolase (59), phosphohexoseisomerase (56, 77), trioseisomerase (105), lactic dehydrogenase (77), ATP generation (77) and glyceraldehyde-3-phosphate dehydrogenase (105) are all apparently equal in the two kinds of cells. That GAPD is active in sensitive cells is significant not only because of its strategic activity in carbohydrate metabolism but because glutathione has been shown to be bound to it and may be a co-factor for its activity (29, 67). In addition, nucleoside phosphorylase (47) has been reported normal. Most investigators have inferred that the activity of 6-phosphogluconolactonase is normal, as indicated, for example, by use of the Glock and McLean (46) procedure for determining the G-6-PD activity (124). Determination of TPN and DPN has revealed an elevation of the former and probably of the latter (103). The reduced forms are apparently correspondingly decreased (Kellermeyer and Schrier, personal communication).

In confirmation of Francoeur and Denstedt (44), we have found that glutathione reductase in hemolyzates can utilize DPNH as well as TPNH, though to a lesser extent (21), and have, therefore, placed glutathione reductase as a pivotal reaction between the glycolytic and oxidative pathways. Determination of the DPN and TPN formed from the respective reduced forms by specific enzymatic assay accounted for more than 90% of each of the reduced forms used and no evidence for the conversion of one to the other was found. The possibility of the existence of two reductases has not been excluded. Nor is it known to what extent, if any, DPNH is utilized by glutathione reductase in intact cells.

Other abnormalities apparently unrelated to carbohydrate metabolism have now been reported. By adhering to the very rapid kinetics of the reaction (72), Tarlov and Kellermeyer (2, 113) have been able to show that catalase is also deficient in primaquine-sensitive erythrocytes, being about 60% of normal. Catalase in human erythrocytes is so active that this deficiency hardly seems sufficient to cause any failure to remove hydrogen peroxide almost instantly. Nevertheless, this enzyme is significant because it is the only enzymatic activity of primaquine-sensitive cells which has been shown to decrease at the time of hemolysis. As with glutathione, the fall is precipitous and, in fact, precedes the change in glutathione. Unlike glutathione, the return to normal is very slow, occupying the life-span of the injured red cells (2). Tarlov has also found an increased tendency of primaquine-sensitive cells to undergo hemolysis when they are incubated in dilute solutions of hydrogen peroxide (personal communication).

Another parameter of this disorder has been demonstrated by Szeinberg *et al.* (109), to be a low incorporation of glycine-C^{14} into the glutathione of sensitive cells. This may be related to the decreased content of glutathione in the sensitive cells. During this investigation, these workers found that the GSH of sensitive cells was also unstable to added cysteine. Omission of this amino acid was necessary in order to obtain a true measure of the glycine-C^{14} incorporation. This effect of cysteine may make difficult further study of GSH synthesis in sensitive cells, *in vitro*, since the formation of γ-glutamylcysteine has been shown to be the first step in the synthesis of the tripeptide (107). Presumably, this substance, like cysteine, might cause the GSH to fall. Studies with glutamine instead of glycine will also require comparison of the activities of a γ-glutamyl transferase reported to be present in human erythrocytes (99). The evidence for synthesis of GSH in human and some animal erythrocytes has recently been reviewed (29).

Investigating still another metabolic area, Tarlov has recently found that, during a primaquine-type hemolysis, the total lipids of the red blood cells decrease sharply, returning to normal in approximately 50 days. This is in contrast to the lipid changes after acute blood loss (2). Brewer (personal communication), who has investigated glyoxalase in both types of erythrocytes, has found no significant differences in the activity of this enzyme. He has, however, pointed out that a consequence of the fall of glutathione would be a failure of glyoxalase activity for which glutathione is required.

Although other abnormalities almost certainly will be found, to date, the most characteristic and severe altera-

tion in erythrocytes bearing the primaquine-sensitive pattern is the deficiency of glucose-6-phosphate dehydrogenase, the first enzyme in the pentosephosphate pathway. Other abnormalities may eventually be interpreted as either compensatory or secondary to this deficiency, although it would be difficult to interpret the decreased activity of catalase on this basis. The increased activities of glutathione reductase and aldolase may be associated with the younger cell age (8, 36) of the erythrocytes in primaquine-sensitive individuals. For, although earlier studies failed to reveal a shortened life-span of primaquine-sensitive red cells, more recent investigations by Kellermeyer and by Brewer (2) have revealed that the life-span of the primaquine-sensitive erythrocytes in males is reduced by approximately 20%. This is in accord with the demonstration by Marks and associates (78) that the younger red cells in human blood have a higher level of glucose-6-phosphate dehydrogenase activity. Thus, primaquine-sensitive individuals are actually undergoing a chronic hemolysis but without anemia.

METHODS

Primaquine-sensitivity is a genetic defect not restricted to the erythrocyte. It is not, in itself, lethal and, in fact, was discovered as an iatrogenically induced effect. Thus, it can and does occur in a large number of people throughout the world so that experimental study of this entity is available to and being carried on by investigators in many laboratories.

So far, few differences among the results from various laboratories have appeared. Most of these can probably be attributed primarily to population or genetic rather than technical variation. However, with much data still forthcoming, not only on the biochemistry of the erythrocyte, but also in relation to other tissues, diseases and biological effects, it is evident that the opportunity for discrepant results to appear which might lead to unnecessary confusion in the literature is increasing. Already, just enough variation in the methods of assay for G-6-PD has been used to make comparison of the degree of deficiency among various populations difficult. The order of the deficiency is apparently between 5–15% of normal in fully affected individuals. Although, in some instances, it is enough to demonstrate a reaction or substance as either present or absent, in these studies careful quantitation is usually necessary because the various parameters must be compared among fully sensitive, intermediately sensitive, and nonsensitive erythrocytes obtained from individuals before, during and after exposure to hemolytic agents. It is hoped that the following abbreviated remarks restricted to human erythrocyte studies will serve to indicate some of the precautions and limitations or advantages of various techniques used.

Preparation of erythrocytes, hemolyzates and stromata. Most experiments require that the erythrocytes first be washed free of plasma. The commonly used solution for this purpose is isotonic sodium chloride, but other isotonic fluids can be used, such as potassium chloride, Krebs-Ringer-Phosphate, etc. For enzyme assays, the choice of washing fluid has not been shown to be critical. However, the repeated centrifugations necessary for complete washing may result in a population of washed erythrocytes of slightly older mean age (8, 78), which could result in lower activity of those enzymes which have been shown to decrease with increasing age of the erythrocyte. For intact cell studies the pH of the suspending fluid is critical (81). In particular instances, special techniques may need to be applied, e.g., to ensure removal of all the white cells. An example of this was in demonstrating that the mature red cell does not synthesize lipids (73). The white cell's ability to synthesize lipids is so many times greater than that of the red cell that even a few remaining white cells were enough to produce the artefact of apparent synthesis of lipids by the red cells (Marks, personal communication). Likewise, in studies of glucose utilization and pentosephosphate formation, intact erythrocytes free of white cells must be used.

Hemolyzates may be prepared by diluting the washed cells with water to cause hypotonic lysis or, rarely, by making the surrounding medium hypertonic, or by rapidly freezing and thawing the washed erythrocytes. None of these methods appear to have given discrepant results with regard to enzyme assays in hemolyzate except with respect to the presence or absence of released stromata or 'ghosts'. Demonstration of the differential inactivation of G-6-PD by stromata can be shown after hemolysis by any of these three methods (20). For demonstration of another effect of stromata, however, hemolysis must be produced by gradual decrease in tonicity according to a recent report (96).

To remove stromata from hemolyzate, centrifugation having force of at least 20,000 × g is necessary up to periods of an hour or more, and to be certain that all stromata are removed the supernatant should be recentrifuged. Higher forces (e.g., 55,000 × g) are not necessary but lower forces are insufficient to remove all stromata. This can be shown either microscopically (using phase contrast) or by comparison of the inactivation of G-6-PD during incubation (unpublished data). Data of experiments in which this precaution was not taken may require re-evaluation. The stromal layer is always fluffy but hemolysis by freezing and thawing gives the sharpest separation on centrifugation, perhaps by splitting lipoproteins.

Enzyme assay in hemolyzates (G-6-PD, 6-P-GD, and GSSG reductase). Two basic methods have thus far been used for estimating glucose-6-phosphate dehydrogenase as well as 6-P-GD and glutathione reductase from erythrocytes.

1) The spectrophotometric method utilizes the changes in absorbancy at 340 millimicra occurring with formation of TPNH from TPN (increase in absorbancy for G-6-PD or 6-P-GD) or TPN from TPNH (decrease in absorbancy for glutathione reductase). In hemoglobin free preparations this is the method of choice except in

special instances where use of the more sensitive spectrophotofluorimetry may be required (65, 66). In the presence of hemoglobin, however, even in an instrument such as the Cary recording spectrophotometer a concentration of only 0.2 gm of hemoglobin/100 ml requires slit widths greater than 2.0 mm. Even so, at this concentration of hemoglobin none of these three enzymes from either primaquine-sensitive or nonsensitive erythrocytes are in sufficient amount to provide maximum activity for their respective reactions in the presence of excess substrate. The rates of reaction at this concentration of hemolyzate are on the order of $1–3 \times 10^{-8}$ M of coenzyme reduced or oxidized per ml reaction mixture per minute at 37°C. (Long and Carson, unpublished data). (The maximum rate which can be provided by hemolyzate at a final hemoglobin concentration of approximately 1.8 gm/100 ml is nearly an order of magnitude higher.) Most of the reports in which this has been the basic method have used much smaller amounts of hemolyzate at room temperature with final hemoglobin concentrations of approximately 0.03–0.06 gm/100 ml giving enzyme concentrations in the first or linear portion of the enzyme concentration curve in the presence of excess substrate.

For determination of G-6-PD in hemolyzates by this method, the use of the Glock and McLean procedure (46) as recommended by Zinkham and Lenhard (124) is to be preferred especially in determining the G-6-PD in hemolyzates from heterozygous blood. Most investigators have used G-6-P only as substrate and their data include activity of 6-P-GD by formation of 6-P-G by 6-phosphogluconolactonase.

2) The second method which has been used in these studies is based on determination of the other product of the reaction catalyzed by glutathione reductase, viz., the GSH formed. Two molecules of GSH are formed for each molecule of GSSG reduced or TPNH oxidized. G-6-PD and 6-P-GD are assayed by coupling with glutathione reductase. This procedure is more tedious than the direct spectrophotometric method since the GSH is determined on protein-free filtrates. The nitroprusside reaction used according to Grunert and Phillips (48) with the precautions described by Flanagan *et al.* (43) is very sensitive. The standard curve in an instrument such as the Beckman B spectrophotometer is linear to an absorbancy of 2.0, representing a range of 2.5 to 50 micrograms of GSH per ml of final solution read on the instrument. Thus, for determination of erythrocyte GSH and GSSG (by difference from total glutathione after electrolytic reduction) (37, 43), the method is ten times more sensitive for example, than iodometric titration. However, when expressed in terms of molecules of TPNH per ml of reaction mixture in the assay of glutathione reductase activity, the range of TPNH determinable is of the same order as direct reading at 340 mμ. The range for the latter is approximately $1 \times 10^{-8}–3 \times 10^{-7}$ M TPNH per ml in a 3.0 ml reaction mixture and a 1 cm light path. The range for the former is approximately $2 \times 10^{-8}–7 \times 10^{-7}$ M TPNH per ml of a 7.0 ml reaction mixture determined as described by Flanagan *et al.* (43).

The advantage in determining GSH instead of TPN is that higher concentrations of hemolyzate may be used for various special purposes: *a*) Using coupled reactions, very small amounts of oxidized coenzyme may be estimated such as in indicating the presence of bound TPN in hemolyzate (22) and, *b*) the presence of any dehydrogenase which will reduce TPN or DPN (or, presumably, any enzymatic activity which can be coupled to a dehydrogenase) can be indicated by reduction of GSSG (because GSH found shows that TPNH or DPNH has been formed); *c*) maximum rate of glutathione reductase can be estimated.

Standards of reference. Because counting red cells is subject to large errors, two standards of reference for enzyme activities of hemolyzates are commonly used, *1*) reference to hemoglobin concentration or *2*) reference to volume of packed erythrocytes.

1) Hemoglobin has been used as a standard of reference because in the non-nucleated erythrocyte it is present in large amounts, is metabolically inert, and can be accurately determined. However, the enzymatic activities are a function of the cell rather than the hemoglobin and in those instances where there is change in size or shape of the cell, e.g., microcytosis in iron deficiency, or macrocytosis in pernicious anemia, reference to this standard will not give accurate results. The practice of correcting enzymatic activities to a given Hgb value can be valid only for activities on linear portions of the enzyme concentration curve (unless the equation of the curve is known).

2) The volume of packed erythrocytes as a standard of reference is also subject to error. Assuming accurate measurement of the volume, it will nevertheless contain a variable percentage of 'trapped' plasma or washing fluid depending on the size of centrifuge tubes and force of centrifugation. This volume will also vary with change in size and shape of the red cells. When necessary, it can be determined by dilution with a nonpermeable substance such as I^{131} labelled albumin.

In summary, both the methods and standards of reference commonly used for assay of enzymes in hemolyzates have been adequate for differentiating fully primaquine-sensitive red cells from nonsensitive cells. However, for purposes such as study of metabolic pathways or synthetic mechanisms, many factors must be re-examined each time a new method is taken up. Possibly, the development of an instrument such as the Coulter red cell counter together with estimations of size and shape of the cells and determination of 'trapped plasma' will enable reference to activity per cell to replace those standards which may give artefactual results.

CHARACTERISTICS OF ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE

The first purification of glucose-6-phosphate dehydrogenase from the red cells of both normal and primaquine-

sensitive individuals using DEAE chromatography was accomplished by Kirkman (63, 64, 65). In both preparations he found the same characteristics of the enzyme with respect to the catalytic site. He found the Michaelis constant (Km) for TPN to be 2.1×10^{-6}, for G-6-P, 3.95×10^{-5}, superimposable pH optima curves and the same heat of activation, 9500 calories per degree per mole. Also, both preparations use the abnormal substrate 2-deoxyglucose-6-phosphate at 9% of the maximum rate for G-6-P with a Km of 6.9×10^{-4}. When subjected to DEAE column chromatography, both preparations show a single peak with identical movement, either alone or when mixed. The quantitative difference, however, persists during the purification procedures.

Kirkman found the purified preparations to be unstable and, of the various protective substances which he tried, the oxidized coenzyme, TPN, protected quite out of proportion to the others in concentrations as low as 10^{-5} M. He suggested that this might be of importance in connection with another phenomenon, the differential inactivation of G-6-PD by erythrocytic stromata, which we had observed.

In hemolyzates inactivation of G-6-PD during incubation at 37° or 45°C is greatly accelerated in the presence of stromata, either from sensitive or nonsensitive erythrocytes (20, 22). Glutathione reductase and 6-phosphogluconic dehydrogenase remain active. This effect of stromata is nondialyzable but heat-labile. In the presence of stromata incubation of hemolyzate from primaquine sensitive cells at 45°C for one hour will reduce the glucose-6-phosphate dehydrogenase activity virtually to 0 (zero). Some activity of the glucose-6-phosphate dehydrogenase in hemolyzates from nonsensitive cells will, however, remain after such an incubation. An exact titration of the comparative activities of stromata from the two kinds of cells has not, however, been accomplished. Presumably, apparent differences in the per cent inactivation or protection of glucose-6-phosphate dehydrogenase activities from this effect are due to the initial quantitative difference of G-6-PD rather than a qualitative difference in the enzymes from the two kinds of cells. TPN, DPN and nicotinamide protect against this effect when added to hemolyzates containing stromata prior to incubation.

Inactivation of glucose-6-phosphate dehydrogenase can also be demonstrated in stroma-free hemolyzates by adsorption of pyridine nucleotides with acid-washed Norit. Incubation of hemolyzate after treatment with Norit again results in loss of glucose-6-phosphate dehydrogenase activity which, in this instance, can be prevented by addition of TPN and DPN but not by nicotinamide. Nicotinamide protects by preventing the pyridine nucleotidase activity of stromata from destroying TPN but TPN and DPN stabilize the enzyme directly. Since DPN in purified preparations does not stabilize G-6-PD (65), this protection by DPN may occur by prior conversion to TPN.[4] Chemically related compounds, such as 6-amino-nicotinamide, nicotinic acid, isonicotinic acid and isonicotinic acid hydrazide have offered no protection. EDTA has often but not invariably given partial protection. Failure of isonicotinic acid hydrazide to inhibit pyridine nucleotidase activity of rat, mouse and rabbit brain or spleen has been reported although it is ten times as effective as nicotinamide against this activity in the corresponding tissues of beef and lamb (120).[5]

After prolonged dialysis of hemolyzate in phosphate buffer or by use of the technique of gel filtration (86), it is possible to demonstrate that TPN is bound. This is shown by the ability of hemolyzate with added 6-phosphogluconate and oxidized glutathione to reduce GSSG without addition of TPN. Because the bound TPN rises and falls concomitantly with G-6-PD activity it has been inferred that TPN is bound to G-6-PD in hemolyzates, as might be expected from the work of Kirkman on purified preparations. The fraction of nondialyzable TPN bound to G-6-PD in hemolyzates has not been determined, however. As a corollary it is also inferred that TPN is probably not bound to 6-P-GD or glutathione reductase of human erythrocytes nor required for their stabilization.

Whether or not this differential inactivation of G-6-PD has physiological significance is not known. However, intact cells incubated in isotonic hemolyzates also inactivate glucose-6-phosphate dehydrogenase of the hemolyzate and hemolyzates of intact cells heated in isotonic sodium chloride, to produce injury without hemolysis, have lower G-6-PD activity and less nondialyzable TPN.

These findings and the unexpected hemolytic action of dimercaprol found by Tarlov have led to trial of nicotinamide, nicotinic acid and EDTA, *in vivo*. Nicotinamide either on a short term basis given concomitantly with primaquine (Kellermeyer, personal communication) or after prolonged daily ingestion (3–4 months) prior to and during administration of primaquine has had no effect on the course of hemolysis in sensitive Negro males (Tarlov, personal communication). Nicotinic acid after 3–4 months administration, however, has been protective on administration of primaquine with the degree of hemolysis being reduced to about 60% of that shown in figure 2 (Tarlov, personal communication). Similar protection was given by daily intravenous

[4] The protection by DPN of G-6-PD in hemolyzates by direct action on the enzyme has not received absolute proof. DPN used (Sigma) contained no TPN by assay with TPN specific yeast G-6-PD. Neither was it active in dialyzed hemolyzates with added G-6-P or 6-P-G as substrate. However, less than 1% contamination of DPN by TPN would suffice to give some protection to the G-6-PD. Neither has direct experimental evidence for formation of TPN from DPN been obtained.

[5] Several years ago we devised a simple test for primaquine-sensitivity based on this phenomenon utilizing both stromata and Norit and have used it successfully in several hundred determinations (Carson, Ickes and Schrier, unpublished data). However, because it distinguishes heterozygous blood no better than other published tests we have withheld it from publication. The Methemoglobin Reduction Test of Brewer *et al.*, which does distinguish heterozygous (intermediately sensitive) blood is preferable (17).

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administration of EDTA during the course of primaquine (Tarlov, personal communication).

Adequate evidence exists demonstrating that, *in vivo*, nicotinic acid unlike nicotinamide, raises the pyridine nucleotides of human erythrocytes (51, 52). *In vitro*, nicotinic acid but not nicotinamide is utilized in the steps leading to synthesis of DPN (88, 89). Similarly, large doses of nicotinic acid but not nicotinamide effectively reduce high blood levels of cholesterol (4). Tryptophan, the amino acid precursor of nicotinic acid and derivatives (30), has also been reported to raise the pyridine nucleotides of human erythrocytes *in vivo* (38). This observation may lead to an explanation for EDTA protection since this compound has been shown to alter human tryptophan metabolism (90). Presumably, the EDTA acts by its chelating properties wherever the site, since it has been reported to be undegraded on excretion (5). In collaboration with Hankes and Brown, initial studies show no difference in the urinary excretion products after administration of a loading dose of tryptophan to male sensitive and nonsensitive subjects. This is not unexpected in the unstressed state.[6]

Recently, in addition to the above results, a report from Israel by Rimon and associates (96) suggests that normal red cell stromata, unlike sensitive stromata, may also contain an activating factor for glucose-6-phosphate dehydrogenase of primaquine-sensitive as well as nonsensitive cells. This became demonstrable by preparing the erythrocyte stromata by dialysis of cells against decreasing concentrations of sodium chloride to produce gradual hemolysis. This discovery will be most significant if it should prove that lack of this factor in primaquine-sensitive cells represents either the primary expression of the genetic defect or its evidence of a genetic variation peculiar to the population under investigation.

Sufficient evidence has now accumulated to indicate that the deficiency of glucose-6-phosphate dehydrogenase is not restricted to the erythrocyte but is lower than normal in other tissues (91–93), probably to a more severe degree in sensitive Caucasians than in the sensitive Negro Americans (76). That the latter may also have a lower level of glucose-6-phosphate dehydrogenase in other tissues than the red cells is indicated *1*) by the recent work of Zinkham (121), who has shown the activity to be lower in the lenses of primaquine-sensitive Negroes; *2*) whole body studies using the apparatus of LeRoy *et al.* (70). Primaquine-sensitive males who have undergone proven hemolysis expire approximately 5% of glucose 1-C^{14} in 2 hours after injection as compared to approximately 10% in nonsensitive individuals (Okita and Carson, unpublished data), and *3*) the inability of the bone marrow of primaquine-sensitive males to synthesize erythrocyte lipids after drug-induced hemolysis although these lipids are synthesized after blood loss from phlebotomy (2). The relationship of G-6-PD deficiency to other physiological and pathological processes, therefore, gains considerable import.

Of great interest is the discovery that there is at least one genetic variant of G-6-PD deficiency different from primaquine-sensitivity. Blood from some cases of congenital nonspherocytic hemolytic anemia was found not only to exhibit GSH instability but also appeared to have either a more severe deficiency or complete absence of G-6-PD as compared to primaquine-sensitive blood (82, 83, 106, 124). These mostly Caucasian patients were undergoing clinical hemolysis without the administration of primaquine or other known hemolytic agent. In one such case, Kirkman *et al.* (66) were able to purify the enzyme and to show that the Km values for G-6-P and TPN were significantly different from purified G-6-PD of nonsensitive and primaquine-sensitive erythrocytes. The values were G-6-P, 2.0×10^{-4} M and for TPN 1.3×10^{-5} M; the abnormal substrate 2-deoxy-glucose-6-phosphate also gave a Km 4–5 times higher than previously purified G-6-PD. Moreover, the pH optimum curve was modified. Nevertheless, the heat of activation and movement on DEAE columns were alike, indicating that the physical difference is subtle. Evidence was obtained suggesting that this qualitatively different enzyme is less stable than the enzyme from nonsensitive and primaquine-sensitive erythrocytes.

MECHANISM OF HEMOLYSIS

For initial clues to the mechanism of hemolysis, we must turn to studies of intact erythrocytes. When erythrocyte respiration is stimulated by methylene blue, the late Dr. E. S. G. Barron demonstrated that primaquine-sensitive cells could easily be distinguished from nonsensitive cells because they failed to respire as effectively. He regarded this as natural proof for activation of the pentosephosphate pathway by methylene blue as well as confirmation of the deficiency of G-6-PD. Johnson and Marks (56) and Murphy (personal communication) have also shown this failure of respiration in intact primaquine-sensitive cells using glucose 1-C^{14} as well. Brin and Yonemoto (18) and Murphy (81) have shown by using glucose labelled with C^{14} in various positions that methylene blue acts by stimulating the oxidative pathway in nonsensitive human erythrocytes.

The abnormality of the pentosephosphate pathway in primaquine-sensitive erythrocytes is further demonstrated by studies of glucose utilization. On addition of methylene blue, primaquine-sensitive erythrocytes, free of white cells, have decreased glucose utilization (fig. 4) (Tarlov, personal communication, and Zinkham, personal communication), although glucose utilization in normal cells is increased. Exactly similar data can be obtained using another dye, phenazine methyl sulfate (Tarlov, personal communication). In analogous studies the formation of pentosephosphate is also decreased in primaquine-sensitive cells but increased in nonsensitive cells, even though total pentosephosphate in freshly drawn blood is equal in the two kinds of cells (59). Thus,

[6] These data using nicotinic acid, tryptophan and related compounds are preliminary. Exploration of this area especially because of its clinical component and possible genetic variations among susceptible populations will probably require several years.

not only does the pentosephosphate pathway fail on addition of methylene blue but, under these conditions, pentosephosphate is not supplied by reversal of the reactions of the nonoxidative limb of the pathway. Added inosine is, however, utilized by primaquine-sensitive cells during these incubations (unpublished data).

Further evidence for failure of the pentosephosphate pathway in primaquine-sensitive cells is to be found in studies of methemoglobin reduction. Gutman, Jandorf and Bodansky (49) demonstrated in 1947 that pyridine nucleotides are required for reduction of methemoglobin. Small amounts of methemoglobin found in human erythrocytes (115) are constantly being reduced, usually by a pathway requiring DPNH. However, stimulation of the pentosephosphate pathway evokes the rapid reduction of methemoglobin by a TPNH mediated pathway.

Ross and Desforges (97, 98) found that primaquine-sensitive cells could not reduce methemoglobin when stimulated with methylene blue. Brewer *et al.* (17) have been able to quantitate this effect (fig. 5) as a test for primaquine-sensitivity. These investigators have found that the rate of reduction of methemoglobin in the presence of glucose and methylene blue can be made proportional to the phenotype of *in vivo* hemolysis and, unlike previous tests, this one correlates well with the intermediate hemolyses occurring in heterozygous females.

The degradation products of primaquine are known to cause methemoglobinemia (58), as are several other drugs which have also caused hemolysis (39–41). Current experiments by Brewer, Tarlov and Alving (personal communication) indicate that the hemolytic degradation products of primaquine and related drugs, like methylene blue, stimulate glucose utilization in nonsensitive cells but not in primaquine-sensitive cells. Szeinberg and Marks (110) have reported that cysteine and ascorbic acid also stimulate the pentosephosphate pathway. Kellermeyer (personal communication) has show that in methemoglobinemia induced with primaquine in normal subjects, the older fraction of the cells contains more methemoglobin than the younger fraction.

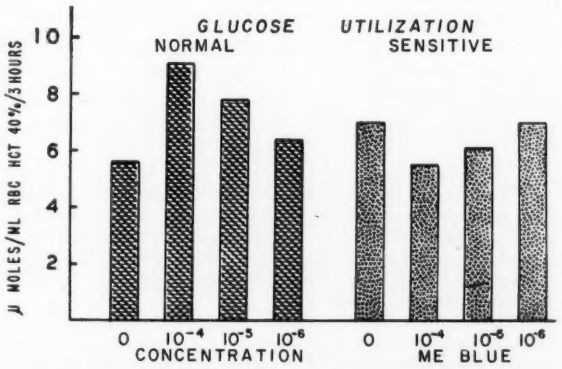

FIG. 4. Effect of methylene blue on glucose utilization of intact normal and primaquine sensitive human erythrocytes (free of leukocytes). Concentration in moles/liter. (Data of Tarlov.)

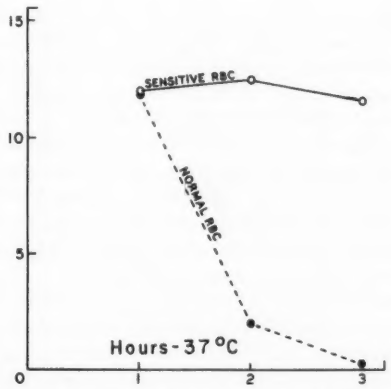

FIG. 5. Effect of methylene blue on methemoglobin reduction in human erythrocytes. Ordinate represents gm methemoglobin per 100 ml. (Data of Brewer.)

Brewer (personal communication) has shown that higher levels of methemoglobin are induced by administration of nitrites in primaquine-sensitive individuals than in normal individuals. Only slightly increased levels of methemoglobin are, however, found during a primaquine-induced hemolysis presumably because the older methemoglobin-containing cells are the ones destroyed.

Methemoglobin, as shown by Austin and Drabkin (3), is in a much more reactive state than hemoglobin because, unlike other forms of hemoglobin, the absorption spectrum of methemoglobin is sensitive to change of pH and ionic strength even though the Fe^{+++} remains tightly bound to the molecule (50, 100). As recently reported by Jandl (55), the process becomes irreversible when the methemoglobin is further converted to sulfhemoglobin which, in turn, apparently coalesces to form Heinz bodies to which GSSG attaches. In an *in vitro* system by which primaquine is degraded after being mixed with blood by exposure to ultraviolet light between 300–400 mμ, it is clearly shown that primaquine-sensitive cells form both GSSG and methemoglobin. Glutathione disappears after being oxidized (unpublished data). This sequence of events is in accord with the recent report of Jandl (55) and with the interpretations of Beutler *et al.* (15), using acetylphenylhydrazine. These changes do not occur in the absence of oxygen. Methemoglobinemia, per se, does not result in hemolysis. Even sulfhemoglobinemia, though irreversible, does not necessarily result in hemolysis and, in fact, disappears as a function of cell age (57). Thus, although these events are apparently established with respect to hemoglobin, for hemolysis the final effect must be on the cell membrane which could occur by an analogous series of reactions affecting membrane sulfhydryl groups and proteins rather than by a subsequent reaction of denatured hemoglobin.

The role of glutathione in the metabolism of the human erythrocyte can be reconsidered with more clarity than heretofore possible. Reduced glutathione is the only freely measurable sulfhydryl containing molecule

in the human erythrocyte. Both reduced and oxidized glutathione are, unlike TPN, almost completely dialyzable from hemolyzates and traces of glutathione can easily be removed after dialysis by use of small amounts of activated charcoal (Norit) insufficient to injure the enzymes. By the technique of gel filtration (86), it is possible to confirm that glutathione is free in hemolyzates by recovering all of the glutathione in the fraction containing the unbound small molecules and, correspondingly, by finding no reduced or oxidized glutathione in the protein-containing fraction (Carson and Zwaanstra, unpublished data). In the stressed primaquine-sensitive erythrocyte, when TPNH production has failed, glutathione reductase ceases to be active and oxidized gluthatione cannot be reduced. Glutathione is in a sense 'unstable' because in the oxidized form it is nonreactive with nitroprusside.

The remaining reduced glutathione could be oxidized either nonenzymatically or enzymatically (29). Mills (79, 80) has suggested that glutathione protects the cell from oxidative processes by itself being oxidized through the action of glutathione peroxidase. This enzymatic activity is, however, very low in human erythrocytes and cannot be demonstrated *in vitro* unless catalase is completely inhibited by large amounts of azide (Tarlov, personal communication). Whether it is physiologically important in human erythrocytes has yet to be shown. Such an indication might be gained by studying its activity in erythrocytes of individuals with another genetic defect, acatalasemia (84). Whether or not the subsequent reactions of GSSG, either with hemoglobin or other proteins, are important in causing hemolysis has not yet been elucidated.

Reduced glutathione, apparently necessary for maintenance of erythrocyte integrity, has at least the following functions in the human erythrocyte: One, is to be a carrier in the pivotal glutathione reductase reaction to provide TPN for the pentosephosphate pathway and possibly under some conditions DPN for the glycolytic pathway. Another, is as a coenzyme itself for glyoxalase (25, 29). A third function, apparently, is direct protection of proteins by providing free sulfhydryl groups more available than those of the proteins which would be injured by attack of these groups (29). Recently, evidence has been reported that this protection is specific for pyrophosphatase not only in hemolyzates of rabbit blood but also in intact rabbit erythrocytes (94). In this protection the necessity for integrity of the pentosephosphate pathway as well as injury of the pathway by stromata was confirmed. In primaquine-type hemolysis the alterations of glutathione, like those of hemoglobin, are evidently secondary to prior events but form an integral part of the hemolytic sequence.

The presence, normally, of small amounts of methemoglobin and oxidized glutathione in human erythrocytes indicates that oxidative processes are present at all times. The equilibrium, however, is usually greatly in favor of reduction, requiring, in the resting state, little activity of the pentosephosphate pathway. In G-6-PD deficient erythrocytes when the pathway is stressed, it cannot meet the challenge and damage by oxidative processes prevails.

At what point the hemolytic agents act, however, is still to be demonstrated. The fact that the activity of the oxidative limb of the pentosephosphate pathway is limited by glucose-6-phosphate dehydrogenase (G-6-PD) deficiency does not necessarily mean that the hemolytic agents block the pathway by acting directly on the deficient enzyme. Even though many agents alter G-6-PD activity of erythrocytes, both competitively and noncompetitively (35), in the *in vitro* system of hemolysis, enzymatic activity of G-6-DP, 6-P-GD and GSSG reductase is not completely destroyed in primaquine-sensitive erythrocytes even after hemoglobin has been converted to methemoglobin and sulfhemoglobin (Carson and Long, unpublished data). Failure of methylene blue and other redox compounds to increase glucose utilization and pentose formation does, however, restrict the site of action to the reactions occurring from the phosphorylation of glucose to the formation of pentosephosphate.[7]

Among the compounds shown by Emerson *et al.* (40), to have hemolytic activity and suggested by them to exert this activity by functioning as redox compounds, is hydroxylamine. Hydroxylamine has long been known to cause Heinz bodies and methemoglobin formation (41). It has not been tested in primaquine sensitivity, but is probably hemolytic. Its reactions in certain biochemical systems related to the present discussion are, therefore, of interest. It has, for example, been used by Cori and Lipmann to demonstrate by forming the hydroxamic acid derivative that 6-phosphogluconolactone is the primary oxidation of product (yeast) G-6-PD activity (27). A product of the reaction is hydrogen peroxide which was removed only if catalase was present. Hydroxylamine has also been shown to replace glycine in glutathione synthesis forming γ-glutamyl cysteinylhydroxamic acid (108). Since hemolytic compounds differ in chemical structure and vary widely in dosage necessary to cause hemolysis (60, 61) it seems unlikely that they are degraded to the same hemolytic product. Therefore, they may act by permitting a hydroxylamine-like reaction with a normally present substance, perhaps a tryptophan intermediate. The 'oxidative processes' normally present in erythrocytes may, in fact, refer to a particular reaction rather than a sum of reactions.[8]

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[7] Theoretically, any mechanism which would block the oxidative limb of the pentosephosphate pathway might allow hemolysis to proceed. This would include as possible primary events binding of glutathione and hypothetical genetic deficiencies of glutathione reductase or 6-P-GD. The present discussion is, however, restricted to primaquine-type hemolysis.

[8] The analogy of these injurious processes to the biological effects of radiation cannot be overlooked. It is a curious coincidence though not pertinent that "For various reasons the root of Vicia Faba has been used by a widening circle of workers and it is probable that today more is known about its response to radiation than about that of any other organism" (95).

Although the available data support such speculation, we may be quite far from elucidating the biochemical steps leading to hemolysis. The erythrocytes of five sheep, by test, have been 'primaquine-sensitive'. Their glutathione was easily oxidizable ('unstable'); their methemoglobin reduction test was positive; their catalase activity was approximately 9% of that in human erythrocytes; and their pentose-phosphate pathway was even less active than in the primaquine-sensitive cells with both their G-6-PD and 6-P-GD being unstable. Nevertheless, the life-span of sheep cells is 120 days (71) and these sheep did not undergo hemolysis *in vivo* even when given large amounts of primaquine, nor did their cells react differently from normal in the *in vitro* system of hemolysis (Carson and Bennett, unpublished data). The most notable biochemical difference between sheep and human cells, as in other ruminants, is their lack of lecithin (which, incidentally, makes them immune to the hemolytic effects of cobra venom) (114). The hemoglobin of sheep cells, which are smaller than human erythrocytes and spherocytic rather than biconcave, differs from human hemoglobin in having much more reactive sulfhydryl groups but neither hemoglobin contains disulfide bonds (7).

Many of the questions raised by discovery of the natural occurrence of G-6-PD deficiency in human erythrocytes are unanswered. During the next ten years investigations utilizing the primaquine-sensitive pattern of biochemical abnormalities may be expected in several areas of biological science. The purpose in this paper, therefore, has been to present a perspective of the developments of the past 10 years associated with G-6-PD deficiency not as a complete review but with an eye to the future in the belief that integration among scientific fields rather than increased specialization may be expected during the next decade.

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American Institute of Nutrition

SYMPOSIUM ON METABOLIC PATTERNS IN PREADOLESCENT CHILDREN[1]

Description of metabolic studies

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RELATIVELY FEW STUDIES have been reported pertaining to the nutritive requirements for preadolescent children. Concerned with the lack of experimental data on the nutritive requirements of this age group, experiment stations in seven southern states have, for the past several years, directed cooperative research efforts to the study of metabolic patterns of preadolescent children.

The purposes of these studies were *a*) to investigate the metabolic patterns of preadolescent children maintained on controlled dietary regimens, *b*) to arrive at more precise standards than now exist for the requirements of selected nutrients for this age group, and *c*) to determine interrelationships of selected nutrients within the metabolic process.

Participating in the studies were agricultural experiment stations from Georgia, Kentucky, Louisiana, Mississippi, Oklahoma, Tennessee, and Virginia, and the Human Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture. Since 1954, four studies have been planned and executed. The methods used in conducting the studies and collecting the experimental data have been described in detail (3).

The first study, in 1954, was planned to determine the metabolic patterns of selected nutrients in preadolescent girls receiving all nutrients at levels recommended by the National Research Council (1). The second study, in 1956, measured the patterns of selected nutrients when intakes of calories and protein were adjusted on the basis of body weight and when the intakes of other nutrients were held at levels recommended by the National Research Council (1). In the third and fourth studies, 1957 and 1958, daily protein intakes were reduced below recommended levels, while intakes of calories and most other nutrients remained at recommended levels (1, 2).

In at least two of the studies, and in many instances for all experiments, metabolic balances were determined for energy, nitrogen, fat, calcium, phosphorus, magnesium, iron, cobalt, copper, manganese, molybdenum, zinc, thiamine, riboflavin, niacin and its metabolites, and vitamin B_{12}.

Certain physical measurements concerned with the nutritional status of the subjects were also made at periodic intervals. A detailed description of all other constituents determined and the results of the hematological studies are published (3).

The experiment conducted in the fall of 1954 ran for 64 consecutive days and consisted of 16 4-day replicate balance periods. The later experiments were conducted during the summer months. The 1956 study ran for 56 consecutive days and consisted of 14 4-day replicate balance periods. The 1957 study consisted of one 4-day balance period where amounts of food eaten were self-selected, followed by four 6-day replicate periods when amounts and kinds of food intake were rigidly controlled. In 1958, the subjects were maintained on the controlled diets for eight 6-day consecutive replicate periods or a total of 48 days. A pre-period of 2 to 3 days preceded the first balance period in each study. At the end of each balance study, experimental conditions were maintained until all fecal markers were recovered.

In addition to the thorough physical examination given to each subject at the time she was selected for participation in the study, physical examinations were also administered at the beginning and end of each study. No illnesses occurred during the study in 1957. In the other three studies the attending pediatricians prescribed medication for some subjects. A detailed listing of medications has been recorded (3).

Means and ranges for initial age, height, and weight

[1] This study was a phase of the Southern Regional Research Project, "Requirements and Utilization of Selected Nutrients by Preadolescent Children," supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946 and the Hatch Act, as amended, and in part by seven southern Agricultural Experiment Stations. The Human Nutrition Research Division of the Agricultural Research was a cooperator in this project.

of the subjects in each study are shown in table 1. In 1954, 1956, and 1958, the ages of all subjects ranged between 6 years and 8 months and 9 years and 7 months. Nine of the ten subjects in 1957 were those studied the previous year and thus the mean age was approximately a year higher than in the other studies. The mean height for subjects used in these studies (130.9 cm) was 2 cm greater, and the mean weight (29.0 kg) was 2 kg greater than the means for the reference child of this age group (2).

When protein intake was lowered in 1957, calcium and phosphorus supplementation was necessary for certain subjects. In 1958, when protein intake was further reduced, supplementation with several vitamins and minerals was required (3). Gelatin was used for 9 of the 12 subjects in the 1956 study to adjust protein intake to body size.

The dietary regime in 1958 was planned so that the energy content of the diet could be increased by 200 calories per subject per day in the event that the minimum daily nitrogen retention (determined by urinary analyses) fell below 0.3 gm per subject. Since this occurred in only one subject, the dietary protein was lowered still further during the course of the study by deletion of fluid milk.

TABLE 1. *Initial Age, Height, and Weight of Subjects in the Four Metabolic Studies*

Year	No. of Subj.	Age		Height		Weight	
		Mean	Range	Mean	Range	Mean	Range
		yr mo	*yr mo—yr mo*	*cm*	*cm*	*kg*	*kg*
1954	11	7 11	7 5–8 7	127.1	122.7–134.0	29.5	22.4–42.7
1956	12	8 4	6 10–9 1	131.0	118.7–145.4	28.5	22.1–33.6
1957	10	9 3	8 7–10 0	137.7	123.2–150.5	31.1	24.7–37.7
1958	13	8 1	6 8–9 7	129.0	120.7–137.2	27.5	21.9–34.1

TABLE 2. *Subjects Identified by Year and by Diet, Duration of Studies and Mean Daily Intakes of Energy and Protein*

Year	Diet No.	Subj. No.	Period No.	No. Days	No. Subj. Days	Mean Intake per Day			
						Energy		Protein	
						Calculated Cal.	Found Cal.	Calculated g	Found g
1954	*1*	1–11	1–16	64	704	1876	1955	60.7	65.3
1956	*2*	12–14	1–14	56	168	1847	1948	48.5	48.1
	3	15–17	1–14	56	168	1846	1966	72.6	72.8
	4	18–20	1–14	56	168	2257	2356	58.6	58.7
	5	21–23	1–14	56	168	2238	2415	88.6	88.2
1957	*6*	12–15, 17, 36	1–4	24	144	2028	2410	37.2	39.1
	7	19, 21–23	1–4	24	96	2247	2545	44.5	44.8
1958	*8*	12, 24–35	1–5	30	390	1978	2178	21.2	22.1
	8′	12, 24–35	6–8	18	234	1896	2235	17.0	18.2
Total		36	Total		2240				

TABLE 3. *Subject Groupings Used to Evaluate Effect of Nitrogen Intake on the Metabolic Patterns of Nutrients*

Group	Year	Subj. No.	Period No.	Nitrogen Intake per Day	
				Total g	Range g/kg body wt.
Between-subject groupings					
*I**	1958	24–35	1–5	3.53	0.104–0.158
*II**	1954	4, 5, 6, 8, 9, 11	1–16	9.67	0.270–0.344
	1956	12, 13, 14, 18, 19, 20	1–14		
*III**	1954	1, 2, 3, 7, 10	1–16	11.60	0.374–0.460
	1956	15, 16, 17, 21, 22, 23	1–14		
Within-subject groupings					
A	1956	12–15, 17, 19, 21–23	1–14	10.89	0.299–0.448
B	1957	12–15, 17, 19, 21–23	1–4	6.66	0.189–0.252
C	1958	24–35	1–5	3.53	0.104–0.158
D	1958	24–35	6–8	2.91	0.085–0.124

* In subsequent papers in this symposium either Roman or Arabic numerals are used to identify these groups.

Table 2 identifies by diet, subject, and study periods the range of protein intake investigated in 59 balance studies (2240 subject-days) with the 36 subjects. The extent to which a given subject served in the various studies can be seen.

For an evaluation of the influence of nitrogen intake on the metabolic pattern, the subjects have been grouped in two different ways (table 3). *Groups I, II,* and *III* were selected for a between-subject evaluation of the effect of nitrogen intake. *Group I* includes the subjects on the low-nitrogen intake, *group II* are those on the medium level, and *group III* are those on the highest level. These three groupings include data on 35 different subjects, and no data have been included for one subject in more than one study.

Groups A and *B* and *groups C* and *D* permit a within-subject comparison of the effect of nitrogen intake on the metabolic pattern. *Groups A* and *B* compare results for nine subjects used in two consecutive years while *groups C* and *D* compare results for 12 subjects in 1958 on two different levels of nitrogen intake.

The manner in which certain other nutrients were influenced by nitrogen intake during the course of these metabolic studies is discussed in the succeeding four papers.

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Nitrogen balance

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MOST OF THE EARLIER STUDIES on nitrogen metabolism in preadolescent children have been concerned with relatively liberal levels of protein intake. Comprehensive summaries of these results have been published in recent years (1–6). In the four metabolic studies reported here daily protein intake was varied from levels somewhat above those recommended by the National Research Council to relatively low levels, that is from as much as 88 g of protein to about 18 g. Results include 59 nitrogen balance studies on 36 subjects; these comprise a larger body of data for children in this age group than have been available previously from a single investigation.

Data for each balance study are listed in table 1, in

TABLE 1. *Nitrogen Balance Data for 36 Subjects on Varying Levels of Protein Intake in Four Metabolic Studies*

Subj. No.	Group	Year of Study	N Intake		N Excretion		N Retention*	
			g/kg b.wt.	g/day	Urine, g/day	Feces, g/day	g/day	intake %
25†	*D*	1958	0.085	2.89	1.82	0.71	0.35	12
34	*D*	1958	0.091	2.88	1.77	0.70	0.41	14
33	*D*	1958	0.096	2.88	1.88	0.69	0.31	11
32	*D*	1958	0.097	2.88	1.73	0.71	0.44	15
31	*D*	1958	0.100	2.87	1.56	0.74	0.57	18
29	*D*	1958	0.101	2.88	1.70	0.59	0.59	20
35	*D*	1958	0.102	3.49	2.33	0.69	0.47	13
12	None	1958	0.103	2.88	1.78	0.67	0.43	15
25	*I* or *C*	1958	0.104	3.52	2.31	0.74	0.47	13
30†	*D*	1958	0.107	2.88	1.79	0.78	0.31	11
28	*D*	1958	0.110	2.83	1.61	0.68	0.53	19
26	*D*	1958	0.111	2.74	1.44	0.71	0.59	25
27	*D*	1958	0.112	2.88	1.73	0.67	0.48	17
35‡	*I*	1958	0.113	3.85	2.71	0.70	0.44	11
34	*I*	1958	0.114	3.52	2.28	0.78	0.46	13
33	*I*	1958	0.120	3.52	2.20	0.68	0.64	18
32	*I*	1958	0.120	3.48	2.22	0.70	0.57	16
24	*D*	1958	0.124	2.88	1.81	0.77	0.30	11
29	*I*	1958	0.126	3.52	2.41	0.61	0.49	14
12	None	1958	0.127	3.52	2.16	0.67	0.70	20
31	*I*	1958	0.128	3.52	1.77	0.67	1.08	31
30	*I*	1958	0.132	3.52	2.46	0.70	0.36	10
28	*I*	1958	0.141	3.50	1.93	0.69	0.88	25
27	*I*	1958	0.141	3.52	2.17	0.65	0.70	20
26	*I*	1958	0.143	3.42	1.65	0.75	1.02	30
24	*I*	1958	0.158	3.52	1.93	0.80	0.79	22
22	*B*	1957	0.189	7.05	6.00	0.58	0.46	7
15	*B*	1957	0.196	5.96	4.32	0.63	1.01	17
23	*B*	1957	0.196	7.13	5.27	0.77	1.09	15
17	*B*	1957	0.198	6.34	5.14	0.73	0.48	8
19	*B*	1957	0.206	7.19	5.26	0.58	1.34	19
21	*B*	1957	0.207	7.30	5.77	0.67	0.86	12
13	*B*	1957	0.221	6.22	4.24	0.81	1.17	19
14	*B*	1957	0.231	6.53	4.72	0.88	0.93	14
36	None	1957	0.236	6.21	4.72	0.74	0.75	13
12	*B*	1957	0.252	6.30	4.00	0.81	1.49	24
8	*II*	1954	0.270	11.22	8.44	1.02	1.76	16
20	*II*	1956	0.289	9.22	7.06	1.08	1.08	12
19	*II* or *A*	1956	0.299	9.47	7.48	0.82	1.18	12
13	*II* or *A*	1956	0.309	7.78	5.66	0.78	1.34	17
14	*II* or *A*	1956	0.311	7.78	5.80	0.88	1.10	14
4	*II*	1954	0.313	10.19	7.78	0.84	1.58	15
18	*II*	1956	0.314	9.47	6.64	1.06	1.77	19
5	*II*	1954	0.321	9.99	7.77	0.96	1.26	13
9	*II*	1954	0.321	11.22	8.50	0.98	1.71	15
12	*II* or *A*	1956	0.335	7.55	5.52	0.87	1.16	15
11	*II*	1954	0.336	11.22	8.32	0.87	2.03	18
6	*II*	1954	0.344	10.08	7.64	0.89	1.55	15
2	*III*	1954	0.374	9.61	7.63	0.87	1.11	12
7	*III*	1954	0.392	11.22	8.36	1.10	1.77	16
3	*III*	1954	0.393	8.82	7.10	0.86	0.86	10
1	*III*	1954	0.403	10.19	7.66	1.20	1.33	13
22	*III* or *A*	1956	0.420	14.12	12.46	0.76	0.90	6§
17	*III* or *A*	1956	0.423	11.63	9.36	0.88	1.39	12
23	*III* or *A*	1956	0.428	14.12	11.47	1.20	1.45	10§
16	*III*	1956	0.428	11.64	9.74	0.89	1.01	9
15	*III* or *A*	1956	0.439	11.64	9.50	0.68	1.46	12
21	*III* or *A*	1956	0.448	14.12	11.98	1.08	1.06	7§
10	*III*	1954	0.460	11.22	8.29	1.08	1.85	16

See table 3, first paper of this symposium, for explanation of groups. * The slight discrepancy between nitrogen retention, corrected for nitrogen losses in excreta, is caused by dropping of decimals for sake of brevity. The original data were processed by electronic computer, IBM 650. † Subjects 25 and 30 failed to store 0.3 g of nitrogen daily during period 6, 1958 study, but stored the levels shown during periods 7 and 8, 1958, when their daily energy intake was increased by 200 Calories. ‡ Subject 35 failed to store 0.3 g of nitrogen daily on the diet containing 3.52 g of nitrogen daily but exceeded this storage level when the daily nitrogen intake was increased to 3.88 g/day during periods 2–5, 1958 study. § Subjects 21, 22, and 23 received about 25% of protein intake as gelatin.

TABLE 2. *Nitrogen Balance and Related Urinary Measurements as Influenced by Nitrogen Intake, Between-Subject Comparisons*

	Unit	Protein Intake		
		Low group I	Medium group II	High group III
		(12)	(12)	(11)
Urine				
pH		5.68	6.36	6.32
Volume	l/day	1.28	0.89	0.90
Creatinine	mg/day	470	574	585
	mg/kg b. wt.	16.84	18.57	21.34
N:Creatinine		4.6	12.7	16.0
Nitrogen				
Intake	g/kg b. wt.	0.128	0.314	0.418
	g/day	3.53	9.67	11.60
Fecal	g/day	0.70	0.92	0.97
Apparent digestibility	%	79.9	90.4	91.6
Urine	g/day	2.17	7.27	9.34
Retention	g/day	0.65	1.47	1.29
Retention	% of intake	18.6	15.1	11.2

No. of subjects are shown in parentheses.

TABLE 3. *Nitrogen Balance and Related Urinary Measurements as Influenced by Nitrogen Intake, Within-Subject Comparisons*

	Unit	1956–57 Studies		1958 Study	
		High group A	Low group B	High group C	Low group D
		(9)	(9)	(12)	(12)
Urine					
pH		6.35	6.09	5.68	5.39
Volume	l/day	1.10	1.36	1.28	1.14
Creatinine	mg/day	552	633	470	479
	mg/kg b. wt.	19.34	19.83	16.84	16.83
N:Creatinine		16.0	7.8	4.6	3.7
Nitrogen					
Intake	g/kg b. wt.	0.378	0.210	0.128	0.103
	g/day	10.89	6.66	3.53	2.91
Fecal	g/day	0.88	0.71	0.70	0.70
Apparent digestibility	%	91.8	89.3	79.9	75.6
Urine	g/day	8.78	4.96	2.17	1.76
Retention	g/day	1.22	0.98	0.65	0.44
Retention	% of intake	12.0	16.1	18.6	16.1

No. of subjects are shown in parentheses.

which results are ranked in order of increasing nitrogen intake per unit of body weight. Each study has been classified in a grouping (see table 3 of first paper in this symposium) in order to facilitate evaluation of the influence of variation in nitrogen intake on metabolic pattern. In the experimental plan, 0.3 g of nitrogen retention daily per subject was decided upon as the minimum level of retention consistent with the demands for body growth in this age group. It is evident that with the three exceptions noted in table 1 all subjects, even those on the lowest level of nitrogen intake, satisfied this criterion for the duration of the studies.

The influence of nitrogen intake on metabolic patterns, based on comparisons between subjects, is presented in table 2. At the low level of nitrogen intake, *group I*, urinary pH was significantly lower than at the two higher levels, *groups II* and *III*. It seems probable that a portion of the shift towards lower pH at lower levels of nitrogen intake was a result of the use of mineral supplementation in the low-protein diet. Furthermore, it seems possible that buffering agents in the urine may have diminished along with the decrease in the excretion of nitrogenous compounds. The urine volumes were adequate for taking care of excretion of metabolic wastes.

Creatinine output diminished along with decreasing nitrogen intake in terms both of absolute amounts and of creatinine per unit of body weight. Ratios of nitrogen to creatinine in urine were strongly and positively correlated with level of nitrogen intake.

As might be expected, fecal nitrogen was influenced relatively little by level of nitrogen intake. However, intake level strongly affected apparent digestibility. Lowered apparent digestibility at the low level of intake may reflect the decrease in proportion of animal proteins in the diet as levels of total protein were lowered.

Excretion of nitrogen in the urine paralleled very closely the level of nitrogen intake. In 1958 when daily determinations of nitrogen in the urine were made, the close relationships between intake and urinary excretion of nitrogen were especially striking. Minor variations in nitrogen content of each of the six menus used within the 6-day balance period were reflected in the urinary excretion of nitrogen.

On the average, subjects on the medium level of nitrogen intake stored slightly more nitrogen than those on the high level. The relatively poor retention observed in the high-protein group may be related to the use of gelatin for augmenting the protein intake of some subjects in this group. This is probably also the explanation for the low efficiency of nitrogen utilization (11.2% of intake) in this group. The most efficient retention occurred in the low-protein group.

The within-subject comparisons for nitrogen balance shown in table 3 represent longitudinal data on two groups of subjects. The within-subject comparisons, as set forth in table 3, might be assumed to provide a somewhat more reliable basis for evaluating nitrogen intake effects on the metabolic pattern than the between-subject comparisons enumerated in table 2. In general, the trends noted in the between-subject comparisons apply equally well to those in within-subjects. Again, the relatively poor efficiency of nitrogen utilization for the subjects in *group A* (table 3) is related to the low retention in subjects in this group who received gelatin.

From these studies it is concluded that 7- to 9-year-old girls will store nitrogen when given a diet low in animal protein sources and providing only 30% of the

presently recommended protein intake. The effect of such low-protein intakes on the metabolic pattern of other nutrients is the subject for discussion in subsequent papers in this symposium.

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Energy

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THE TERM ENERGY, as used in this report, refers to energy balance as determined by bomb calorimetry, which is a true measure of the metabolizable energy of foods. This method involves gross energy determination on food, feces and urine.

The metabolizable energy is defined as the amount of energy available for use after gross food energy has been corrected for losses in urine and feces. Apparent absorption of energy is derived by correcting food energy by the amount excreted in the feces.

Rosenthal and Allison (8) have emphasized that the retention of nitrogen in the animal is in part a function of the energy intake. Allison (1) has stated: "Caloric intake is a significant consideration in the study of every branch of nutrition, but a proper balance between energy and nitrogen metabolism is particularly important to the welfare of the individual."

Munro (5) has stated: "It is generally recognized that nitrogen balance is influenced by energy content of the diet but the extent of the relationship is less widely understood. . . . It is the relationship between energy intake and energy requirements which seems to be the determining factor in influencing protein metabolism."

In the present studies, data on energy balance have been collected over a wide range of protein intake. The daily energy intakes ranged from 1800 to 2600 Calories and the daily protein intakes from 88 to 18 g. The subjects have been grouped according to the criteria set forth in the first paper of this symposium and the procedural details for each study have been described (9).

The mean daily energy intakes for *groups 1*, *2* and *3* varied less than 115 Calories (table 1). In *group 1*, the energy intake was constant at a level of 2167 Calories per day, except for one subject who went into negative nitrogen balance at this level and then received 2286 Calories daily. The variations in total energy intake in *groups 2* and *3* were much greater; the ranges were 1888 to 2370 and 1808 to 2415, respectively. Variations in individual energy intake (Cal/kg of body weight) are shown graphically in figure 1. The group means differed by 10 Cal/kg of body weight. The average weight gains (g/subject/day) were 30, 12 and 12, respectively, for *groups 1*, *2* and *3*. In both *groups 1* and *2* there was one subject who lost weight. These were the heaviest girls in their groups. They weighed 34.0 and 41.7 kg, as compared with means of 28.0 and 30.8 kg for *groups 1* and *2*, respectively, and as compared with 27.0 kg for the reference child (6). The weight losses of these two subjects averaged 10 and 20 g daily on respective energy intakes of 64 and 48 Cal/kg/day. The recommended energy intake is 77 Cal/kg/day (6). With these few exceptions, the energy intakes of the subjects in these

TABLE 1. *Mean Caloric and Nitrogen Intake for Three Groups*

Intake	Group 1 (Low Protein)	Group 2 (Med. Protein)	Group 3 (High Protein)
Cal/day	2,177	2,072	2,063
Cal/kg	79.0	68.6	74.2
Range, Cal/kg	64–97	48–79	69–82
Protein, g/day	22.2	59.8	72.8
Protein, g/kg	0.80	1.98	2.66
Nitrogen, g/kg	0.128	0.316	0.426
Range, g/kg	0.104–0.157	0.270–0.347	0.390–0.458

The slight differences between some figures in this paper and corresponding values in the first two papers in this symposium are due to rounding of machine calculations.

studies could be judged more than sufficient for their needs.

The urinary energy loss increased with the protein intake (table 2), a confirmation of earlier work (2). This was true, whether calculated on a daily basis, unit body weight basis, or percentage of intake basis (fig. 2). The differences between groups were highly significant. The percentages excreted on the two higher protein intakes are of the same magnitude as those found by Hawks (2) for slightly higher intakes of protein (2.9 and 3.5%). Macy (4) reported values of 4% for children of this age group.

Fecal energy loss was comparatively constant (table 2) and ranged from 3 to 5% of the intake. In terms of Cal/day, the difference between group means was less than 12 Cal (fig. 3).

It has been stated (3, 4) that energy in the excreta is about equally divided between urine and feces. In these studies the ratio of urine Calories to fecal Calories was below unity on the low and medium protein intake and approached unity on the high protein intake (table 2).

Judging from these data, the dietary would have to provide above 2.7 g of protein/kg of body weight before the ratio of energy loss via the kidney and intestinal tract would equal unity.

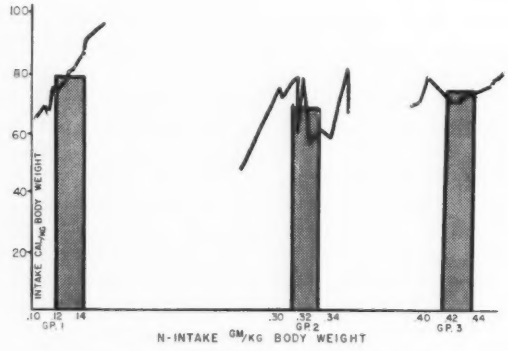


FIG. 1. Relation of N-intake to caloric intake.

TABLE 2. *Energy Excretion on Three Levels of Nitrogen Intake*

	Group 1 (Low Protein)	Group 2 (Med. Protein)	Group 3 (High Protein)
Urinary excretion			
Cal/kg	1.1	2.0	2.7
% of intake	1.4	3.0	3.6
Fecal excretion			
Cal/kg	2.7	2.8	3.0
% of intake	3.4	4.2	4.0
Nitrogen balance			
g/day	0.66	1.42	1.21
Ratios			
Cal/N, intake	617	217	174
Cal/N, urine	14.8	8.6	8.0
Urine/feces, Cal	0.43	0.73	0.97

The slight differences between some figures in this paper and corresponding values in the first two papers in this symposium are due to rounding of machine calculations.

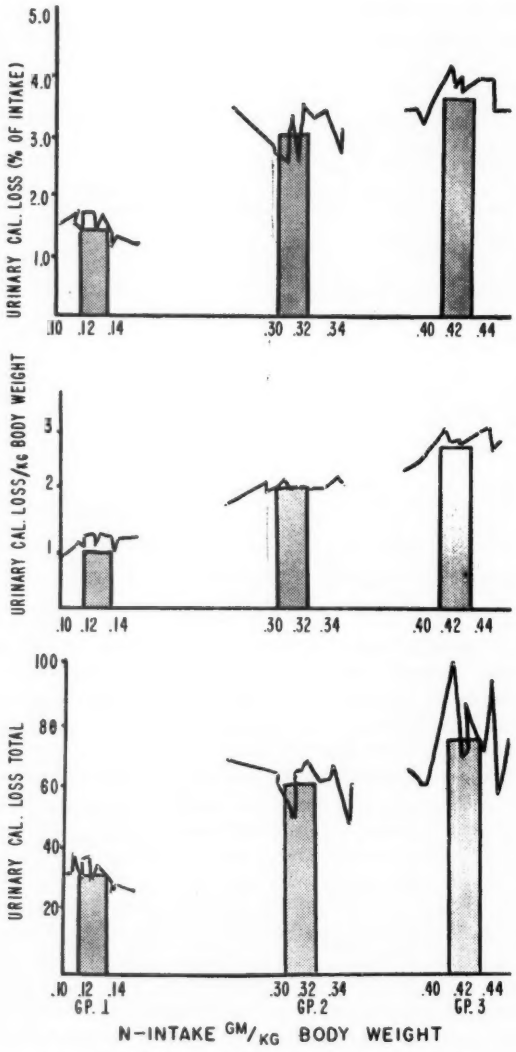


FIG. 2. Relation of N-intake to urinary caloric loss.

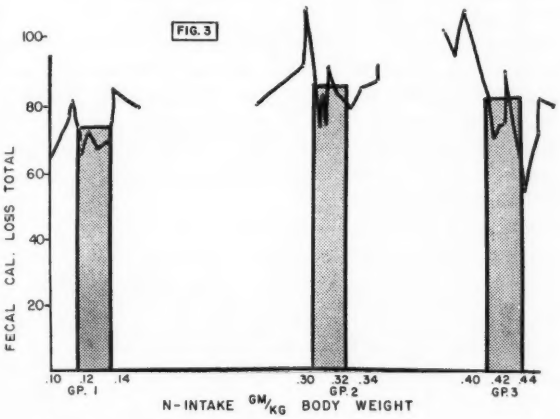
The total energy loss in the excreta ranged from 98 to 180 Cal/day. The mean percentage losses for *groups 1, 2* and *3* were 5, 7 and 8%, respectively.

The difference between the mean energy intake and mean fecal loss was 96%, regardless of nitrogen intake/kg of body weight or Cal intake/day. This value (96%) is within the range of values (94.3 to 97.1%) previously reported for children (2–4, 7, 10). Since the weights of the children varied, the apparent absorption of Cal/kg of body weight differed significantly.

With an increase in protein intake, the percentage of energy available for body needs decreased (fig. 4). The retention values were 95, 93 and 92%, respectively, for *groups 1, 2* and *3*; these correspond with respective retentions of 75, 64 and 69 Cal/kg of body weight. When all the energy data were considered, the same relation

to nitrogen intake was found. The averages for Calories retained were 95, 93 and 91% with respective intakes ranging from 0.085 to 0.20 g, 0.20 to 0.30 g, and 0.30 to 0.45 g of nitrogen/kg of body weight. These observations confirm those of Wang and associates (11) who used somewhat higher protein levels.

In any study of energy, the distribution of energy among the body's needs is of interest, especially in the case of growing children. The mean basal metabolic heat production of the subjects in the three groups increased with increasing protein intake (table 3). This increase was observed in terms of Cal/day and % of intake. The energy need for specific dynamic effect of food eaten



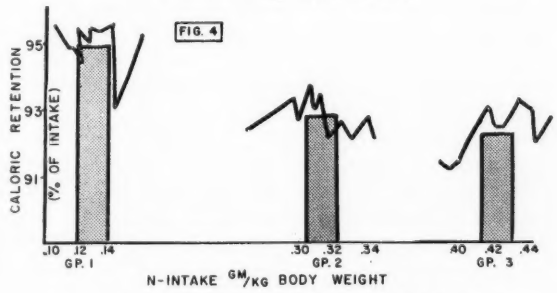


FIG. 3. Relation of N-intake to fecal caloric loss.
FIG. 4. Relation of N-intake to caloric retention.

TABLE 3. *Average Daily Energy Exchange of 35 Subjects, Calculated Values*

Distribution of Calories	Group 1 (Low Protein)	Group 2 (Med. Protein)	Group 3 (High Protein)
Potential food energy, Cal	2,177.0	2,072.0	2,063.0
Potential energy loss in urine and feces, Cal	104.5	148.2	158.0
% of gross Calories	4.8	7.2	7.6
Physiological fuel value, Cal	2,072.5	1,924.0	1,905.0
per kg body weight	75.2	63.8	68.5
% of gross Calories	95.2	92.8	92.3
Basal heat production, Cal	1,069.0	1,094.0	1,108.0
per sq. m. surface area, Cal	44.9	43.8	46.0
% of gross Calories	49.1	52.8	53.7
% of net Calories	51.8	56.9	58.2
Specific dynamic action of food, Cal	124.0	115.0	114.0
Energy available for activity, Cal	879.5	715.0	683.0
% of gross Calories	40.4	34.5	33.1
% of net Calories	42.4	37.2	35.8

was calculated as 6% of the Calories metabolized. Subtracting this calculated energy need and the energy needed for basal metabolism from the physiological fuel value of the diet, the Calories remaining for activity and growth have been estimated. It is clear that the estimated energy available for activity and growth was about 23% less at the high protein level than at the low protein level.

SUMMARY

Energy balances of 35 preadolescent girls on three levels of nitrogen intake have been presented. *1*) As the dietary nitrogen increased, the percentage of energy retained for body needs decreased from 95 to 92. *2*) A highly significant decrease in urinary energy occurred along with the decrease in nitrogen intake. *3*) Energy loss through the feces was fairly constant. *4*) The estimated energy available for activity and growth was about 23% less in the subjects receiving the high protein diet than in those receiving the low protein diet.

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Calcium, phosphorus and magnesium

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THAT UTILIZATION OF CALCIUM, phosphorus and magnesium is improved in adults by high levels of dietary protein has been reported by McCance and associates (8), and Kunerth and Pittman (5, 10). Increased retention was attributed to increased absorption of the minerals from the intestinal tract. The amino acids lysine and arginine have been shown to promote absorption of Ca^{45} in the rat (13). However, in the preschool children studied by Hawks *et al.* (3) calcium absorption and retention were not influenced by an increase in daily protein intake from 3 to 4 gm per kilogram of body weight; absorption of phosphorus was slightly less but retention was increased at the higher protein level. In the work reported here, the influence on metabolism of calcium, phosphorus and magnesium in preadolescent children exerted by variations in daily protein intakes between 0.5 and 3.0 gm per kilogram of body weight was studied.

A detailed description of the subjects, diets and design of the experiments may be found in an earlier publication (11). The first paper in this symposium summarized the plan of the cooperative studies and explained that, expressed as nitrogen intake per unit of body weight, the daily protein intake of each of the 35 children studied falls into one of three distinct levels, the groups designated *I*, *II* and *III*. In this report of the influence of varying levels of protein upon metabolism of calcium, phosphorus and magnesium, discussion will be confined to the pattern of their metabolism in these three groups and in two matched *groups A-B* and *C-D* (the within-subject comparisons). The data presented are mean values for all groups; the procedures for collection of the data have been described (11).

In each of the studies, dietary levels of calcium and phosphorus were planned to meet recommended allowances (9) and to be identical in all diets. Primary calcium phosphate was added to supplement the amounts from foods in the protein-restricted diets. Mean levels of calcium determined by analysis varied from 0.94 gm in *group D* to 1.13 in *group B* and of phosphorus from 1.12 to 1.28 gm per 24 hours in *groups B* and *D*, respectively. Fortified milk supplied vitamin D except in the low-protein diets in 1958 when a supplement of Drisdol provided 400 IU daily. If the calcium and phosphorus content of the diets in all the studies may be assumed adequate for preadolescent girls, the small variations in intake from one study to another would seem too small to materially influence the pattern of their metabolism. The same assumption cannot be made in the case of magnesium since no supplementary source of magnesium was added, and the level in the low-protein diets was considerably below that in the diets meeting National Research Council (NRC) recommended protein allowances. For this reason the pattern of magnesium metabolism will be considered separately from that of calcium and phosphorus.

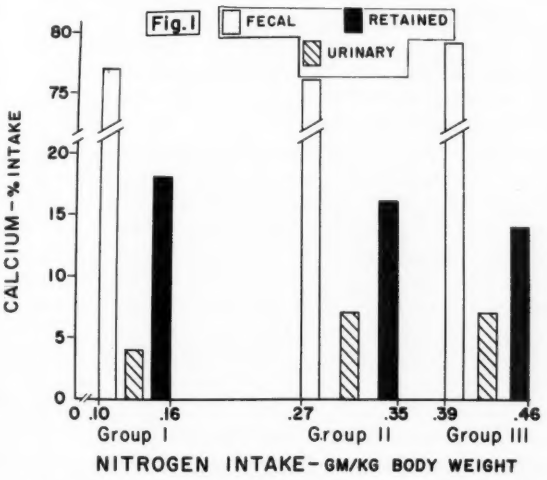
CALCIUM AND PHOSPHORUS

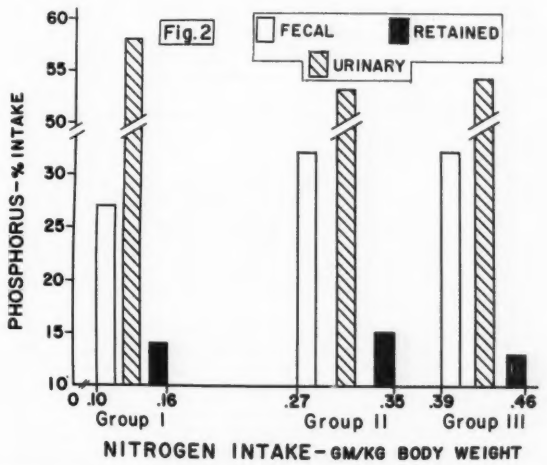
Fecal excretions of calcium by the children in *groups I, II* and *III* were essentially the same (table 1) and represented 77–79% of the intake (fig. 1). Just how much of this fecal calcium represents unabsorbed dietary calcium and how much is endogenous is not known. Leitch and Aitken (6) state that the old idea of no appreciable endogenous fecal calcium excretion in children is no longer tenable and suggest that between infancy and adolescence endogenous loss contributes something like 40 to 60 mg to the daily fecal calcium. Since the present studies do not afford data for estimating endogenous fecal excretion, the term 'absorption' as used in this paper will represent the apparent absorption of calcium, phosphorus and magnesium obtained by simple difference between intake and fecal excretion. On this basis, the amounts of calcium absorbed by the three

TABLE 1. *Calcium and Phosphorus Balances in Groups I, II and III*

	I	II	III
	Calcium (gm/day)		
Intake	1.006 ± .023*	1.010 ± .079	1.046 ± .073
Feces	.772 ± .069	.773 ± .113	.818 ± .094
Urine	.045 ± .024	.074 ± .036	.071 ± .031
		II-I†	III-I†
Retention	.188 ± .063	.162 ± .051	.156 ± .066
	Phosphorus (gm/day)		
Intake	1.257 ± .018	1.164 ± .055	1.245 ± .098
Feces	.341 ± .054	.373 ± .065	.408 ± .037
			III-I‡
Urine	.727 ± .057	.618 ± .068	.668 ± .071
		I-II‡	I-III†
Retention	.188 ± .046	.173 ± .047	.169 ± .036

* Standard deviation of mean. † $P < 0.05$. ‡ $P < 0.01$.





FIGS. 1 and 2. Percentages of dietary calcium and phosphorus excreted and retained. Range of individual nitrogen intakes per kilogram of body weight within each group is spanned by the bars representing fecal, urinary and retained calcium or phosphorus of that group.

groups were the same and were not influenced by differences in level of dietary protein.

Phosphorus absorption was not altogether independent of differences in the diets. Fecal excretions by *groups II* and *III* were similar (table 1) and in each case averaged 32% of the intake. In *group I* fecal phosphorus dropped to 27% of the intake (fig. 2) although phosphorus intake was almost identical with that of *group III*. Phosphorus absorption was significantly higher on the low-protein diet of *group I* than in *groups II* and *III* ($P < 0.01$ and 0.05, respectively). In the matched groups the same trend was apparent, 7 of the 9 subjects in *group A-B* and 8 of the 12 in *group C-D* excreting slightly less fecal phosphorus at the lower protein intake. Increase in phosphorus absorption with decreasing dietary protein was similar to that found by Hawks and her associates in preschool children (3).

Urinary calcium excretions by all three groups were small (table 1). Excretion by *group I* subjects, however, was considerably lower than that of the children receiving the higher protein intakes. Knapp (4) observed that increase in protein intake, without a simultaneous change in dietary Ca:P ratio or increase in acidity of ash of the diet, was accompanied by a small but consistent increase in urinary calcium. Similar findings by McCance and co-workers (8) and Kunerth and Pittman (5, 10) were attributed to increased calcium absorption in adults. In the present studies, mean calcium absorptions by the three groups of children were almost identical; Knapp, also, found no relationship between level of urinary calcium and its absorption or retention in children.

In the case of phosphorus, urinary excretion by the subjects in *group I* was higher than in either of the other groups (table 1). However, *group I* children were those who absorbed the largest amounts of phosphorus (fig. 2), and the higher output may have been due to the larger amount of phosphate entering the circulation. In fact, excretions by the three groups represented 79, 78 and 80% of that absorbed. Excretions in the matched groups also varied with the phosphorus absorbed and gave no indication that storage was influenced by changes in the diets.

Since calcium absorption was independent of the variations in diet and urinary excretions in all cases were so small that the decrease in output on the low-protein diet had little effect on net balance, mean retentions in all three groups were much the same. It is evident (fig. 1) that the changes in dietary protein had no significant influence on calcium retention in the children studied. Increased absorption of phosphorus by the subjects receiving the low-protein diet was nullified by the rise in urinary excretion (fig. 2) and phosphorus retention, also, was independent of the differences in the diets fed.

MAGNESIUM

Interpretation of the pattern of magnesium metabolism at the various levels of dietary protein is complicated by the fact that as the daily protein intake fell below the recommended level the intake of magnesium decreased also. The high-protein diets received by *groups II* and *III* each supplied a mean magnesium intake of 232 mg per day. Although this is less than the 300 mg said to represent the daily magnesium requirement of 7- to 8-year-old children (1, 2) the amount of magnesium in diets meeting NRC recommended allowances should be adequate, as was pointed out by Stearns (12) for well balanced diets in general. The low-protein diet of *group I* in 1958 supplied only 136 mg of magnesium daily, however, and in the final periods of the study reduction

TABLE 2. *Magnesium Metabolism in Groups I, II and III and in Matched Groups A-B and C-D*

Group	Nitrogen Intake gm/kg/day	Magnesium (mg/day)				
		Intake	Fecal	Absorbed	Urinary	Retained
I	.10–.16	135.6 ±2.5*	55.6 ±5.6 III-I†	80.0 ±6.2 II-I†	65.4 ±7.9 II-I†	14.6 ±4.1
II	.27–.35	231.7 ±12.7	133.3 ±22.7	98.4 ±17.9	84.8 ±13.4	13.7 ±8.5
III	.39–.46	231.4 ±10.8	129.2 ±10.8	102.2 ±15.3	92.5 ±14.8	10.4 ±6.4
A-B‡						
A	.30–.45	225.0 ±10.7	118.8 ±12.4	106.2 ±16.8	92.8 ±16.5	13.7 ±8.5
B	.19–.24	176.7 ±7.4	80.5 ±9.3†	96.2 ±14.5	83.8 ±11.3	12.5 ±7.7
C-D§						
C	.10–.16	135.6 ±2.5	55.6 ±5.6	80.0 ±6.2	65.4 ±7.9	14.6 ±4.1
D	.09–.12	120.8 ±4.4	43.2 ±6.2†	77.6 ±8.6	61.4 ±6.7	16.2 ±8.6

The slight differences between some figures in the N intake in this paper and corresponding values in the first two papers in this symposium are due to rounding of machine calculations. * Standard deviation of mean. † Difference significant at 1% level. ‡ Matched group of 9 subjects compared at two different levels of nitrogen intake. § Matched group of 12 subjects compared at two different levels of nitrogen intake. *Group C* is the same as *group I*.

in dietary nitrogen to 0.1 gm per kilogram was accompanied by a decrease in magnesium to 121 mg daily.

The mean intake, excretion, absorption and retention of magnesium in *groups I, II* and *III* and in the matched *groups A-B* and *C-D* are given in table 2. The range of individual nitrogen intakes per kilogram of body weight is included for clarity. On the maximum magnesium intake provided in these diets subjects in *groups II* and *III* excreted in the feces more than half the magnesium ingested and absorbed 42–44% of the intake. In *group I*, with an intake of only 136 mg, fecal excretion was less than half that of the others and 59% of the dietary magnesium was absorbed. The total amount absorbed was, however, significantly lower than in the groups receiving almost double the intake. In each of the matched groups the smaller decrease in magnesium intake was again accompanied by a significant decrease in fecal excretion, resulting in absorption of essentially the same amounts of magnesium. These data suggest that fecal excretion of the mineral is a function of intake, permitting remarkably constant magnesium absorption over a considerable range of intake.

Urinary excretion of magnesium by *group I* subjects was lower than that of *groups II* and *III* and represented 82% of the amount absorbed as compared to excretion of 91% of the absorbed magnesium in *group III*. In the matched groups, although differences were small, all but two of the 21 subjects excreted slightly less magnesium on the more restricted diet. Urinary excretions in *groups II* and *III* were similar to those found by Macy (7) in children receiving an average daily magnesium intake of 297 mg. It might be argued that in *group I* the magnesium intake was so low that some critical level in the tissues had been reached except that the same tendency in urinary excretion was noted in *group A-B* subjects on daily intakes of 225 and 177 mg and in *group C-D* receiving 136 and 121 mg.

As would be expected from these data, retention of magnesium by *group I* children fed the low-protein, low-magnesium diet was equally as good as that in the other groups.

SUMMARY

Patterns of metabolism of calcium, phosphorus and magnesium in 35 preadolescent girls receiving varying levels of dietary protein were compared. Calcium and phosphorus intakes were constant within the limits of experimental error. Magnesium levels decreased when the protein content of the diets fell below the recommended level.

Under the conditions of the experiments storage of calcium, phosphorus and magnesium was independent of the level of protein in the diets. Healthy, well-nourished 7- to 9-year-olds were able to maintain positive balances with respect to these minerals for a period of 7 weeks on diets furnishing only 17 to 20 gm of protein daily. Retention of magnesium was independent of its level in the diet. Although the changes were too small to significantly alter retentions, the patterns of metabolism of calcium, phosphorus and magnesium were altered when the subjects were fed the protein-restricted diets.

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Minor minerals and B-vitamins

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THIS PAPER BRINGS TOGETHER SOME OBSERVATIONS on the effect of nitrogen intake on the output of six minerals —Zn, Mn, Mo, Fe, Cu, Co—and five B-vitamins—thiamine, riboflavin, niacin, and folic and pantothenic acids.

Previous papers have explained how the level of nitrogen intake was regulated in the different diets, and how the intakes of some nutrients such as iron, thiamine, and niacin, were maintained at approximately National Research Council (NRC) levels through supplementation. Intakes of other nutrients were not regulated. Folic and pantothenic acids for example followed nitrogen intake up and down as they would in ordinary diets; the intake of others, such as a few of the minerals turned out to be fairly uniform in most of the diets.

For summarizing the data on these nutrients it was necessary to keep the original diet groupings by years because of variations from diet to diet, and the fact that analyses for these minerals and vitamins were not made in 1957 nor for minerals in 1954. The design of the three studies, the characteristics of the subjects and diets used, and the responses to levels of nitrogen intake are summarized in table 1.

MINERALS

Findings on minerals are available for only 2 years, 1956 and 1958. Table 2 brings together data on intake and fecal output of minerals. The figures shown are averages for the group, per person per day, for all metabolic periods combined. Urinary output is omitted for purpose of this paper because it usually amounted to less than 3% of the intake. It is included for molybdenum because it was half or more of the intake.

Zinc. Zinc was one of the minerals of which output was most affected by nitrogen intake. In 1956, zinc intake varied somewhat with the level of nitrogen intake and average fecal zinc was about 75% of the intake at each level of nitrogen on the four diets. Thus, within the range of nitrogen intake used in 1956, no effect of dietary nitrogen on zinc output was demonstrated.

In 1958, on a much lower nitrogen intake, around 0.1 gm/kg of body weight, the zinc intake was comparable to the lowest levels in 1956. But fecal zinc decreased as the 1958 study progressed over the 48 days and averaged only about half as much as in 1956, as the 12 girls on *diet 8* for 30 days were continued on the same diet with milk omitted for 18 more days. Compared to 75% in the 1956 study, fecal excretion in 1958 was only 54 and 37% of the zinc intake on *diets 8* and *8'*, respectively. The result was a high retention of zinc, both absolute and relative, averaging one-half or more of the intake in 1958.

The relation of fecal output of zinc to nitrogen intake is illustrated in figure 1 by combining data from *diets 2* and *4*, and from *3* and *5*, and comparing with those from *diet 8*. The abscissa represents the nitrogen intake per kilogram of body weight as used in previous papers; the line above each bar shows the level of zinc intake. The height of the bars shows clearly the lower fecal output on the lower nitrogen diets of 1958, compared to those of 1956 with comparable zinc content.

Molybdenum. In 1956, total urinary and fecal excretion of molybdenum was around 95% of the intake and showed no consistent relation to nitrogen intake in the range of 0.3 to 0.4 gm/kg used (table 1).

In 1958 on the low nitrogen diets, total output was less than that in 1956, even though the molybdenum intake was similar in *diet 8*. For example, the total excretion on *diet 8* was only 45% of the intake, compared to 94% on *diet 5* in 1956. The net result was a retention ten times as high in 1958 when the molybdenum intake was most comparable to that in 1956.

Excretion of molybdenum as related to nitrogen intake per kilogram of body weight is shown in figure 2, in which data from *diets 2* and *4* and from *diets 3* and *5* in 1956 are combined as was done for zinc. Fecal and urinary excretion of molybdenum as well as total output are shown separately in this figure because urinary output was over half the intake and was about twice the fecal output. When nitrogen intake was reduced to about 0.1 gm/kg, both urinary and fecal excretion of molybdenum was reduced, and the total output was about half that on the intermediate and higher nitrogen levels in 1956.

Manganese. The effect of the low nitrogen diet on manganese output was toward decreased fecal output as found for molybdenum and zinc. As may be seen from the pairs of data in table 2, manganese intake in 1956 rose slightly with nitrogen intake, but the effect on output could not be attributed wholly to the nitrogen intake. However, in 1958, fecal manganese was lower while dietary manganese remained constant when nitrogen was lowered, and was comparable to the two highest

TABLE 1. *Characteristics of Diets and Subjects Used in 1954, 1956, 1958**

Year, Location, Diet	Planned Level N/kg	Subject				Nitrogen Intake† Found			Nitrogen Retention‡		
		No.	Average age	Initial weight		Total	Per kg		Total	Average N/kg	% of intake
				Average	Range		Average	Range			
	g		*Yr. Mo.*	*kg*	*kg*	*g*	*g*	*g*	*g*	*mg*	%
1954 Fall											
Diet 1–Tenn.	.3–.4	6	7 10	27.3	22–33	9.8	.36	.31–.45	1.29	47	13
Diet 1–La.	.3–.4	5	8 2	32.3	24–43	11.2	.36	.27–.46	1.83	57	16
1956 Summer, Va.§											
Diet 2 (S)	.3	3	8 4	24.0	22–25	7.7	.32	.31–.35	1.20	50	16
Diet 4 (L)	.3	3	8 3	30.8	30–31	9.4	.30	.30–.31	1.34	41	11
Diet 3 (S)	.4	3	8 3	27.0	26–27	11.6	.43	.42–.44	1.28	50	14
Diet 5 (L)	.4	3	8 5	32.3	31–34	14.1	.43	.42–.45	1.14	35	8
1958 Summer, Va.											
Diet 8	.1	12	7 11	27.5	22–34	3.5	.13	.10–.16	0.66	24	19
Diet 8'	.1	Same	8 0	28.4	23–34	2.9	.10	.09–.12	.45	15	16

* Details of diets and subjects described in Metabolic Patterns in Preadolescent Children. 1. Description of Metabolic Studies, Southern Cooperative Series Bulletin No. 64, September 1959. † The slight differences between some figures in this paper and corresponding values in the first two papers in this symposium are due to rounding of machine calculations. ‡ Data based on analyses of collections for each metabolic period in 1954 and 1956, daily in 1958. § 1956 diets adjusted for small (S) and large (L) girls.

TABLE 2. *Mineral Intake and Output by Preadolescent Girls (Average Total Daily**)

Year, Location, Diet	Zinc		Molybdenum		Manganese		Iron		Copper		Cobalt	
	Intake	Feces	Intake	Urine and Feces	Intake	Feces	Intake	Feces	Intake	Feces	Intake	Feces
	mg	*mg*	*mcg*	*mcg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mcg*	*mcg*
1956–Va.												
Diet 2	6.5	4.9	72	64	2.1	1.9	6.8	6.5	1.12	1.12	13.3	8.9
Diet 4	7.6	5.7	75	71	2.8	2.3	7.5	8.1	1.20	1.21	14.5	10.6
Diet 3	9.0	6.7	74	67	2.2	1.8	7.2	6.7	1.14	1.12	13.7	9.5
Diet 5	9.2	7.1	81	76	3.0	2.6	7.9	7.6	1.33	1.32	17.9	11.5
1958–Va.												
Diet 8	7.2	3.9	80	36	2.9	2.1	9.8	8.5	1.06	1.19	8.6	7.2
Diet 8'	6.6	2.5	55	35	2.9	1.9	10.8	8.7	1.06	1.08	8.0	7.1

* Mineral analyses were made on collections for even-numbered metabolic periods in 1956, all periods in 1958.

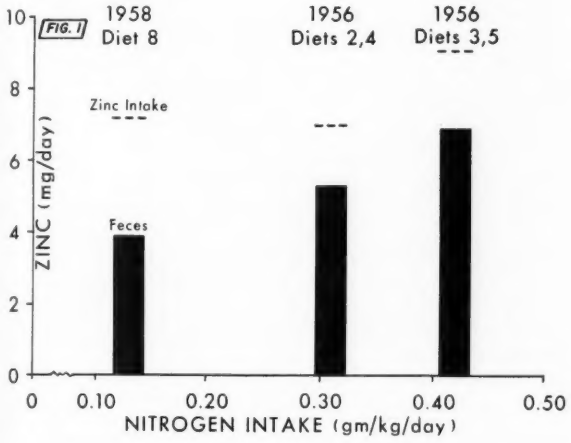
1956 diets (*diets 4* and *5*). The manganese output averaged less than 70% of the intake on the two 1958 diets compared to around 85% on higher nitrogen diets in 1956 which contained comparable amounts of manganese. The lower fecal elimination of manganese in 1958 resulted in a retention two to three times that on comparable intakes in 1956.

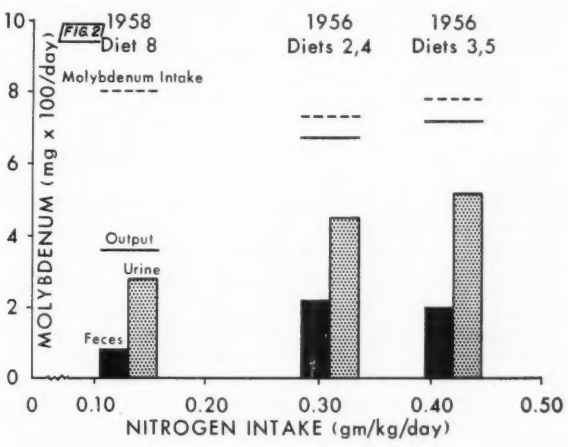
Iron. The data for iron indicate that on a low nitrogen diet the amount of iron in feces, like zinc, molybdenum, and manganese, tends to be reduced and thus retention increased. Findings in 1956 were inconclusive, however, because dietary iron was less than 8 mg/day, and fecal iron averaged 96, 94, 108, and 96% of the intake on the four *diets*, *2*, *3*, *4*, and *5*, respectively. With balances so close to equilibrium, it may be that iron intake was a limiting dietary factor in 1956 and this obscured effect of nitrogen intake.

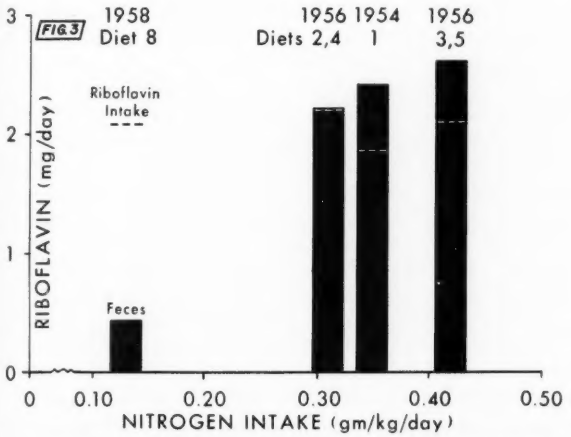
In 1958, when dietary iron was 2 to 4 mg higher than in 1956, fecal output was only 1 to 2 mg higher, and iron retention was two to three times as much on the low nitrogen diet of 1958 as in 1956. Studies of iron elimination at different levels of nitrogen intake with iron intake held constant throughout might show whether iron output on low nitrogen diets is decreased in a degree similar to that found for zinc and molybdenum.

Copper. The effect of nitrogen intake on copper output was inconclusive because in 1958 the copper intake as well as the nitrogen was reduced. The 1956 diets resulted in equilibrium between average intake and outgo, whereas the 1958 diets resulted in negative balances about equivalent to the reduction in copper intake. Hence, copper intake rather than nitrogen intake may have dominated the response.

Cobalt. For cobalt there was no indication of a tendency to decreased output on a low nitrogen intake as found for other minerals, but the evidence is inconclusive due to variations in cobalt intake. In 1958, cobalt intake was about half as much and fecal output was about two-thirds as much as in 1956; hence output was not reduced in the same proportion as intake. Expressed as







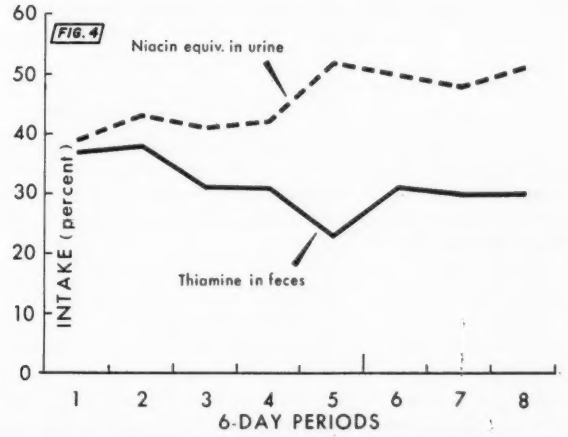


FIG. 1. Relation of nitrogen intake to zinc intake and fecal output in preadolescent girls.

FIG. 2. Relation of nitrogen intake to molybdenum intake and output in preadolescent girls.

FIG. 3. Relation of nitrogen intake to riboflavin intake and fecal output in preadolescent girls.

FIG. 4. Effect of duration of low nitrogen diet in 1958 on fecal thiamine and urinary niacin equivalent in preadolescent girls.

percentages of the corresponding intakes, the output was around 60% for small girls (*diets 2* and *3*) and 70% for large girls (*diets 4* and *5*) in 1956, and about 85% on the two 1958 diets.

B-VITAMINS

The effect of levels of nitrogen intake on output of the various B-vitamins was more variable than on output of the minerals. Data for all 3 years are summarized in table 3 for the vitamins other than niacin, and in table 4 for niacin.

Riboflavin. Among the B-vitamins analyzed, data on riboflavin presented the most clear-cut evidence of effect of low nitrogen intake. With riboflavin intake fairly uniform, both urinary and fecal riboflavin tended to be lower on the low nitrogen diets of 1958 than on the high nitrogen diets of 1954 and 1956 (table 3). Among the two pairs of 1956 diets for small girls (*diets 2* and *3*) and for large girls (*diets 4* and *5*) with riboflavin intake fairly constant, total excretion tended to be lower on the diets lower in nitrogen. In 1958, with dietary riboflavin generally as high as in 1956 and even higher than in 1954, fecal riboflavin was only about one-fifth as much. This may reflect a lowered intestinal biosynthesis of riboflavin on the low protein diets, or increased riboflavin requirements due to an altered protein metabolism, or other dietary factors may be involved.

The depressing effect of nitrogen intake on fecal output of riboflavin may be seen in figure 3 based on combined data for 1954 and for the two pairs of diets in 1956. The riboflavin intakes were comparable in all 3 years, but on the high nitrogen diets riboflavin in feces exceeded intake. Note that the depressing effect of lower nitrogen intake was relatively more marked at 0.1 gm/kg level than in the range of 0.3 to 0.4 gm/kg.

Thiamine. Among the four 1956 diets, when thiamine intakes were fairly similar, urinary thiamine was con-

TABLE 3. *Vitamin Intake and Output by Preadolescent Girls (Average Total Daily*)*

Year, Location, Diet	Riboflavin			Thiamine			Folic Acid		Pantothenic Acid	
	Intake	Urine	Feces	Intake	Urine	Feces	Intake	Urine	Intake	Urine
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mcg*	*mcg*	*mg*	*mg*
1954 Fall										
Diet 1–Tenn.	2.0	1.2	2.1	0.45	.30	.34	97	1.08	4.7	2.6
Diet 1–La.	1.7	1.4	2.8	1.06	.29	.41	99	1.45	4.2	3.1
1956 Summer, Va.										
Diet 2	2.0	1.1	2.2	.64	.15	.25	79	1.07	4.2	1.4
Diet 4	2.4	1.2	2.2	.64	.20	.28	80	1.07	4.8	1.6
Diet 3	2.0	1.2	2.6	.80	.25	.26	76	1.05	5.4	2.0
Diet 5	2.2	1.3	2.6	.71	.28	.38	85	1.32	5.6	1.9
1958 Summer, Va.										
Diet 8	2.1	1.0	.4	1.03	.24	.33	50	1.41	3.0	1.4
Diet 8'	2.2	1.0	.4	1.01	.23	.30	54	1.41	2.4	1.1

* Analyses for riboflavin and thiamine were made on collections from odd-numbered metabolic periods in 1954 and 1956,. all periods in 1958; analyses for folic and pantothenic acids are for even-numbered periods in 1954 and 1956, all periods in 1958.

TABLE 4. *Niacin Intake and Urinary Excretion of Niacin Metabolites by Preadolescent Girls (Average Total Daily*)*

Year, Location, Diet	Intake—Potential Niacin			Urinary Excretion					
	Niacin	Tryptophan equiv.†	Total	Niacin equiv.‡	Nicotinic acid	N'methyl nicotinamide	6-pyridone	Quinolinic acid	Tryptophan
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
1954 Fall									
Diet 1–Tenn.	10.4	11.8	22.2	10.9	.29	3.2	9.6	5.3	8.9
Diet 1–La.	10.6	13.9	24.5	12.7	.30	3.3	11.6	5.3	9.1
1956 Summer, Va.									
Diet 2	7.7	9.2	16.9	5.9	.17	2.6	4.2	4.4	4.9
Diet 4	9.2	11.1	20.3	7.4	.26	2.6	5.9	5.7	5.9
Diet 3	9.9	14.3	24.2	9.7	.21	3.0	8.5	3.7	6.0
Diet 5	10.7	14.7	25.4	7.7	.31	2.3	6.6	3.0	7.9
1958 Summer, Va.									
Diet 8	12.5	3.2	15.7	6.8	.28	2.5	5.3	3.2	7.5
Diet 8'	12.2	2.3	14.5	7.2	.29	2.9	5.3	3.2	5.9

* Analyses made on collections from even-numbered metabolic periods in 1954 and 1956, all periods in 1958. † Equivalent in niacin calculated as 1/60 of tryptophan. ‡ Niacin equivalent of nicotinic acid, N'methyl nicotinamide and 6-pyridone combined, excluding quinolinic acid and tryptophan.

siderably lower on *diets 2* and *4* of lower protein content than on *diets 3* and *5* (table 3). Likewise, in 1958 with low dietary nitrogen, urinary thiamine was relatively low despite a high thiamine intake. Both urinary and fecal thiamine were lower on the low nitrogen diet of 1958 than on the 1954 *diet 1* La., which had a similar thiamine but much higher nitrogen content. However, the effect of nitrogen intake on output or perhaps biosynthesis of thiamine is uncertain because the vitamin intake was not controlled.

Folic Acid. Urinary folic acid was only a small proportion, under 3%, of the intake (table 3), and was fairly uniform on the diets in 1954 and 1956 except on *diet 1* La., and *diet 5*. The low nitrogen diet in 1958 provided only two-thirds as much folic acid as did diets in 1956, but urinary output was even higher than in 1956. There was a strong trend among individual children toward uniform output by the end of the study. These responses may reflect a depletion of body reserves of folic acid on the curtailed intake, or be an indirect effect of the limited nitrogen intake. Data on fecal folic acid might have been useful.

Pantothenic acid. Findings on pantothenic acid in relation to nitrogen intake are inconclusive, apparently because of a seasonal or situation effect. Within a given study, urinary excretion of pantothenic acid followed intake, but differed considerably between studies even on diets of similar pantothenic acid content. In the fall of 1954 the urinary excretion averaged about 65% of the intake in contrast to 33% on comparable intakes in the summer of 1956. In the summer of 1958 on the low-protein diet urinary excretion was 48% of the intake; it was as much in actual amount as in 1956 on diets furnishing more pantothenic acid and nearly three times as much nitrogen. On the low nitrogen 1958 diet there was a consistent downward drift in urinary output and a strong trend to uniform excretion among individuals by the end of the study, as was observed for folic acid.

Niacin. It might be anticipated that niacin more than other B-vitamins would be affected by the amount and kind of dietary protein. Niacin requirement is known to be affected by dietary protein through tryptophan. Whether the relationship is the same for growing children who are

synthesizing protein as for adults is not known. Little is known about how characteristics of dietary protein other than tryptophan content affect niacin metabolism. Evaluation is complicated by the need for considering many niacin metabolites in urine. Subject differences among the girls, in the narrow age range studied, were greater in excretion of niacin metabolites than in the excretion of any other B-vitamin. This suggested that several nutrition and metabolic factors may be involved.

Data in table 4 show that in the three studies the niacin intake was more uniform than was the potential amount from tryptophan. Using factor 1/60 for conversion of tryptophan, niacin and tryptophan furnished approximately equal shares of the total niacin potential of the diet in 1954 and 1956; but in 1958 four to five times as much came from niacin as from tryptophan. Diets in 1958 were highest in niacin alone, although lowest in tryptophan and nitrogen. Diets in 1956 were mostly the reverse—lowest in niacin, highest in tryptophan and nitrogen.

Despite the wide difference in the nitrogen and niacin content of the diets, urinary excretions of niacin metabolites like pantothenic acid were more alike in the summers of 1956 and 1958 than in the fall of 1954. However, when expressed as percentage of niacin potential intake, niacin equivalent excretion amounted to 50% in 1954, 35% in 1956, and 46% in 1958. Niacin equivalent excretion was low on the low niacin *diets 2* and *4* of 1956, as well as on the low potential niacin intakes of 1958. The low excretion on *diet 5*, which was highest of the 1956 series in total nitrogen, tryptophan, and potential niacin, may have been due partly to the amount of gelatin (19 gm/day) given to increase the nitrogen content of the diet. This will be discussed later.

Data on the excretion of the various metabolites show that the similarity of the 1956 and 1958 outputs, contrasted to 1954, was due to the two largest fractions, N′methyl nicotinamide and mostly 6-pyridone.

Urinary quinolinic acid and tryptophan were not included in the computations of urinary niacin equivalent, but excretion of these also differed in the three studies. Excretion of quinolinic acid on the intermediate nitrogen intake (*diets 2* and *4*) in 1956 was similar to that on 1954 diets. Excretions on the high nitrogen intake (*diets 3* and *5*) were lower, similar to those on the low-nitrogen low-tryptophan diets of 1958. During the course of the diets an upward trend in the excretion of total niacin equivalent, when expressed as percentage of intake, was noted in all 3 years but it was most marked in 1958. Urinary tryptophan excretion was fairly uniform over the duration of the 1954 diets, but showed a downward trend on the 1958 diets when intake was low.

One criterion of nutritional adequacy or deficiency used in international surveys of nutritional status is the ratio of milligrams of N′methyl nicotinamide to grams of creatinine in urine. A ratio between 1.6 and 4.3 is considered an acceptable range for adequacy in adults. In these three studies of 35 girls for a total of 268 subject-periods, the ratio was below the acceptable range for only two periods. If one classifies the ratios for each period using categories of under 3, 3 to 6, and over 6, the 1956 study had the most ratios under 3 (17%); and the 1958 study the most ratios over 6 (40%). In other words, the highest ratios occurred on diets lowest in nitrogen, tryptophan, and niacin potential, although they were highest in niacin alone. Relatively low urinary creatinine on the 1958 diets explained most of the high ratios.

Effect of protein quality. Reference has been made to the amount of gelatin in *diet 5* in 1956. This diet also had the highest tryptophan content as well as the highest nitrogen, 14 grams. The low excretion of niacin equivalent on *diet 5*, despite the high intake, might be interpreted to mean a higher niacin requirement in the presence of much dietary gelatin or a higher niacin utilization in the presence of abundant dietary tryptophan. On this *diet 5*, excretion of both quinolinic acid and N′methyl nicotinamide were the lowest of all studies, even lower than on the low-nitrogen low-tryptophan diets of 1958 (table 4). *Diet 5* resulted in the lowest total nitrogen retention in 1956, and also in the highest urinary output of thiamine, riboflavin, and folic acid. *Diet 5* also altered the pattern of excretion of niacin metabolites; compared to *diet 3* of similar N/kg content, it resulted in at least a third more urinary nicotinic acid and tryptophan and about a fourth less of each of the three other components; compared to *diet 4* with similar total niacin equivalent excretion, deviations were in the same directions but more marked for quinolinic acid and less marked for other components. These observations suggest the need for further investigation of the effect of the nature of protein on requirements and utilization of various B-vitamins.

Protein and intestinal biosynthesis. The possible role of intestinal biosynthesis as affected by protein was suggested previously by the reduced fecal riboflavin observed on the low nitrogen diets in 1958. Conversely, the highest fecal riboflavin occurred on diets highest in total nitrogen (*diets 1* La., *4*, and *5*) (tables 1 and 3). Tendencies to reduced fecal thiamine as the study progressed in 1954 as well as in 1958 may indicate in part some failure of the constant diets used to support the usual intestinal biosynthesis. However, antibiotics were administered to some children in both years. Some of the lowered urinary excretion of B-vitamins may also have resulted from a curtailed supply from biosynthesis, due to antibiotics, low protein, and perhaps to other factors as well.

Deviations in metabolism in periods 4 and 5 in 1958 also indicate that intestinal biosynthesis played a role. Fecal outputs of thiamine and riboflavin were less when elevated body temperatures appeared in some children and antibiotics were given by the physician. In the same periods the fecal output of copper, and to some extent that of manganese and zinc, was greater, indicating poorer absorption or increased metabolic loss. On the other hand, urinary riboflavin was increased in periods 4 and 5 and urinary niacin equivalent was highest in period 5. The high average outputs were traceable mostly to the girls who had received the medications but all

girls did not respond alike. These trends may reflect an increased demand during elevated body temperatures as well as some effect of the antibiotic on intestinal biosynthesis.

Figure 4 illustrates the effect of periods 4 and 5 on urinary niacin equivalent in contrast to fecal thiamine when both are expressed as percentage of intake. It illustrates also the opposite upward and downward trends in urinary and fecal excretions of these two vitamins on a low nitrogen diet, even though distorted by the situation in periods 4 and 5. During these periods fecal thiamine and urinary pantothenic also were depressed. These unexpected responses complicated evaluations of the influence of nitrogen intake. They do suggest that the effects of infections and antibiotics on many nutritional requirements deserve further investigations.

SUMMARY

The main effects of nitrogen intake on the metabolism of certain minerals and vitamins in 7- to 9-year old girls under the conditions of these three studies may be summarized as follows: The outputs of zinc and molybdenum, and to some extent manganese and iron, tended to be lower on lower nitrogen diets than on higher nitrogen diets with comparable mineral content. The net effect was higher retentions of these minerals on low nitrogen diets. Outputs of iron and copper showed some indications of lower fecal output on the lowest nitrogen diets, but results were inconclusive because of variations in intake of minerals.

Among the B-vitamins studied, fecal riboflavin was most affected by the low nitrogen intake. The fecal output was greatly depressed even though intake remained fairly constant, perhaps indicating a stimulating effect of protein intake on intestinal biosynthesis of riboflavin. Urinary thiamine tended to be lower, folic acid higher on the lowest nitrogen intakes. On one diet containing much gelatin to increase the nitrogen content total nitrogen retention was low, the urinary output of niacin metabolites, particularly N′methyl nicotinamide, 6-pyridone, and quinolinic acid was lower, and that of thiamine, riboflavin, and folic acid was higher than on similar diets containing little or no gelatin.

American Institute of Nutrition

SYMPOSIUM ON NEW ASPECTS OF NUTRITION UNCOVERED IN STUDIES WITH IRRADIATED FOODS

A long-term feeding study of irradiated foods using rats as experimental animals[1]

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STUDIES HAVE BEEN CARRIED OUT in our laboratory since 1954 on the nutritive properties of irradiated foods. Three phases of this investigation are described in this report: *1*) growth and reproduction in rats receiving an irradiated synthetic diet; *2*) growth, reproduction and gross and histopathologic data on three generations of rats receiving various combinations of control and irradiated boned chicken and green bean diets; and *3*) congenital blindness in young from mothers receiving various diets.

EXPERIMENTAL

The rats used in all tests were from a colony which has been maintained without the introduction of any new strain at the Texas Agricultural Experiment Station for more than 30 years. This colony has been in the Department of Biochemistry and Nutrition since March 1946. The first study was carried out to determine the effect of an irradiated synthetic diet on growth, reproduction, and life span of rats. In the reproduction study, 20 females received irradiated and control diets. In addition, 10 females and 10 males were used to determine the effect of the irradiated diet on the life span. Parent, F_1, F_2, and F_3 generations were observed in both the reproduction and longevity studies.

Diet. The composition of the synthetic diet and the procedures for treating the diets were described previously by Richardson and Brock (3).

A second long-term study was carried out to determine the nutritive properties of irradiated green beans and boned chicken. Each food was treated with zero, 2.79 and 5.58 megarads of gamma rays. The frozen and irradiated foods were furnished through the Office of the Surgeon General. The irradiated and nonirradiated control green beans and chicken were delivered in sealed No. 10 tin cans. The control foods were stored at approximately 0°F until prepared for the feeding tests. The irradiated foods were stored at ambient temperature (70–80°F) for a minimum of 3 months before they were used in feeding trials. Some of these foods have been stored for over 2 years and their physical characteristics and general appearance are the same as when these were first used.

All treatments of green beans were heated in an oven in a covered stainless steel pan at 325°F for 1 hour. The boned chicken was heated in the same way at the same temperature for 2 hours. After cooling, the foods were run through a sausage grinder and each food was mixed in the diet at a level of 35% on a dry matter basis. Foods were cooked and fresh diets were mixed twice per week. Thirty per cent dry weight of the green bean-chicken diets was a synthetic diet. The composition of this portion was the same as that described by Richardson and Brock (3) with the exception that the mineral mixture was increased to 8.0%, and 1.0% of calcium carbonate was added.

The following amounts of vitamins in milligrams were added to 100 g dry weight of diet: thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, 1.0 each; calcium pantothenate, 4.0; niacin, 2.0; folic acid, 0.1; biotin, 0.02; crystalline vitamin B_{12}, 0.001; choline chloride, 200.0; inositol, 10.0 and menadione, 0.75. Each rat received orally one drop of a mixture of oleum percomorphum, alpha tocopherol and corn oil (Mazola) twice weekly. One drop of this mixture supplied approximately 300 IU of vitamin A and 0.25 mg of alpha tocopherol. The final green bean-chicken diets supplied ap-

[1] The experiments reported here were undertaken under Contract No. DA-49-007-MD-582 with the Office of The Surgeon General. The opinions expressed are those of the authors' and are not necessarily endorsed by the Department of the Army.

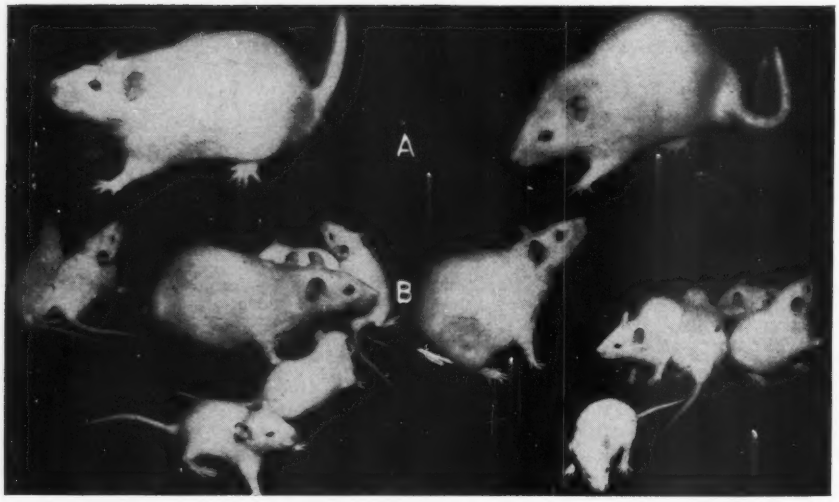

FIG. 1. Animals on left received the control and those on the right received the irradiated synthetic diet. *A*) F_3 generation males. *B*) F_2 generation females and 18-day-old young in sixth litter of each female.

proximately the following amounts of macronutrients: protein, 33.0 %, fat, 20.3 %; ash, 5.8 %; calcium and phosphorus 1.0 % each. The calculated approximate caloric value was four or more per gram.

The nutritive properties of green beans and chicken were tested simultaneously using a factorial design recommended by the University of Chicago Statistical Research Group and concurred in by the Food and Drug Administration. Nine different combinations of the green beans and chicken were used. The combinations used are shown on the charts and in the tables.

The parent generation contained a total of 220 animals and the F_1 and F_2 generations contained 132 animals each.

RESULTS

Irradiated synthetic diet. The results on growth, reproduction, and longevity of rats receiving an irradiated and control synthetic diet were reported by Richardson and Brock (3). Photographs of F_3 generation males and F_2 generation females and their sixth litter of young are shown in figure 1*A* and *B*.

In general, rats receiving the irradiated diets grew at slightly slower rates than those receiving the control diet, but both groups eventually attained the same weight. Likewise, mothers receiving the irradiated diet weaned a slightly smaller percentage of their young than those on the control diet, but these differences were very small and they are probably not due to the nutritive value of the diet.

A microphthalmia (an abnormality in which one or both eyes failed to develop) occurred in a few young from mothers receiving both kinds of diet. In a few tests, this abnormality was slightly higher in the young from mothers receiving the irradiated than it was in the young from those receiving the control diet. This eye abnormality will be discussed further in the section headed 'Congenital Blindness.'

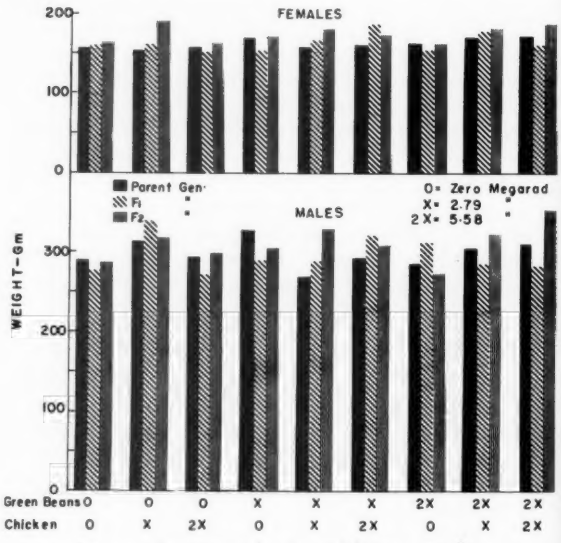

FIG. 2. Average gain in weight—12 weeks.

Irradiated green beans and boned chicken. The criterion used to measure the nutritive performance of the irradiated green beans and boned chicken were growth, fertility, number of young born and weaned, and hematologic and gross examination of all animals in the parent, F_1 and F_2 generations. Hematologic studies were made also on two young from each litter in the F_3 generation. Tissues of all animals in the parent generation were prepared for histopathologic examination and a detailed histopathologic study was made of animals receiving the control foods and those receiving single and combined foods that had been irradiated at 5.58 megarad dose. The average gain of the males and females at 12 weeks in the parent, F_1 and F_2 generations are given in figure 2.

The average gains in weight at 12 weeks; hemoglobin,

hematocrit, white cell count, lymphocytes, polymorphonuclear neutrophils; and weights of heart, lungs, liver, spleen, kidneys and testes at autopsy of all rats in the parent, F_1 and F_2 generations receiving the irradiated and control green bean-chicken diets were put on IBM cards and analyzed by analyses of variance at the Texas A & M Data Processing Center. The only significant difference was in weight of sex.

Fertility and reproduction. The percentage of fertile males and females was as high in groups receiving irradiated as in those receiving the control foods. The poorest reproduction record was in the F_1 generation rats that received green beans irradiated at the 2× level and control chicken. Six males and six females received this diet and only two each were fertile. Since all the animals were fertile in most of the groups when both foods were irradiated, the poor performance of the animals in this group was not attributed to the irradiated green beans. The percentage of young weaned in the various groups ranged from 12 to 98. In some cases the percentage weaned was higher when both foods in the diet were irradiated than when both foods were control. In view of these results, the variation in the young weaned between the groups receiving the various diets was not attributed to the irradiated food.

F_3 generation young from mothers receiving both foods irradiated at the 2× level were as well developed as young from those receiving control foods. The young from mothers receiving the green bean-chicken diets were no different in appearance than those shown in figure 1*B*.

CONGENITAL BLINDNESS

All animals used in the irradiated food study were observed for any abnormality which developed. The only abnormality that repeated itself was microphthalmia, hereafter referred to as blindness, which developed in the young from mothers receiving the control diet as well as in the young from those receiving the irradiated diet. However, in the first investigation, the incidence was consistently higher in groups receiving the irradiated diet. The data for four generations receiving the synthetic diet in the original trial are summarized in table 1.

Hain (1) has reported that the incidence of microphthalmus in her stock rats was approximately 2.47 %

TABLE 1. *Incidence of Blindness in Young Weaned from Females Receiving a Control and Irradiated Synthetic Diet*

Generation	Control			Irradiated		
	Total No.	Blind		Total No.	Blind	
		No.	%		No.	%
Parent	767	21	2.7	856	49	5.7
F_1	639	7	1.1	732	44	6.0
F_2	750	13	1.7	587	39	6.6
F_3	510	2	0.39	389	5	1.3
Total	2666	43	1.61	2564	137	5.34

TABLE 2. *Blind Young Weaned from Females* that Were Descendant of the Same Parent Female*

Parent	Diet	Females, No.	Young		
			Weaned	Blind	
			No.	%	No.
A	C	12	430	2.79	12
	I	23	686	7.29	50
B	C	15	457	1.97	9
	I	12	462	4.76	22
C	C	28	1029	1.55	16
	I	20	625	7.20	45
D	C	13	474	0.42	2
	I	0	0	0	0
E	C	0	0	0	0
	I	13	451	2.22	10
F	C	10	251	0.80	2
	I	0	0	0	0
G	C	0	0	0	0
	I	10	292	3.42	10
H	C	1	23	4.35	1
	I	2	48	0	0
I	C	1	2	0	0
	I	0	0	0	0
Total	C	80	2666	1.58	42
	I	80	2564	5.34	137

* F_6, F_7, F_8, and F_9 generations combined. C = Control. I = Irradiated.

TABLE 3. *Blind Young Weaned from Females in Three Different Tests*

Trial	Diet	Females Observed, No.	Young Weaned		
			Total	Blind	
				No.	%
Original*	C	80	2666	43	1.61
	I	80	2564	137	5.34
1st Repeat	C	50	1127	7	0.62
	I	50	877	14	1.60
2nd Repeat	C	50	1425	24	1.68
	I	50	1223	21	1.72

* Females in this trial were same as those shown in table 2. C = Control. I = Irradiated.

in 5,000 animals, whereas King (2) found about 3.5% in a stock of Wistar strain animals. This defect appeared to be a hereditary condition, but the mode of inheritance was not straightforward. However, the data were consistent with the possibility of a dominant factor with a poor expression.

In view of these reports, the ancestry of the females used in the irradiated food study was traced to determine whether the mothers receiving irradiated diets had the same ancestry as those receiving the control diet. It was possible, from the records available, to show that the parent generation females in the irradiated food study were the F_6 generation from the original parent. Therefore, the four generations used in the irradiated food study were F_6, F_7, F_8, and F_9 generations from the parent. The parent female and the number of descend-

ant females that received the control and irradiated foods together with the observation on the young are summarized in table 2.

Again the incidence of blindness was higher in the group receiving the irradiated than in that receiving the control diet even when the mothers were descendants of the same parent female. In view of these results, it was decided to repeat the studies with an irradiated synthetic diet in an attempt to determine the relation of the irradiated diet to the incidence of blindness in the young. Two separate repeat trials were run, and each trial was composed of 50 females that received the control diet and 50 that received the irradiated diet. These data, together with those obtained in the original trial, are summarized in table 3. They indicate that irradiation of the diet does not increase the incidence of blindness. The percentage of blind young weaned from females receiving the irradiated diet in both repeat trials was approximately the same as that weaned in the control groups in the original and second repeat trials. The difference between the control and irradiated groups in the first repeat is due to the normal variation in incidence of blindness in the colony.

TABLE 4. *Blind Young Weaned from Females Receiving Diets not Associated with Irradiated Foods*

Diet	Females Observed, No.	Young Weaned		
		Total No.	Blind	
			No.	%
Synthetic diets				
1	19	191	3	1.57
2	22	488	4	0.82
3	29	384	33	8.59
4	33	438	29	6.62
5	34	385	14	3.63
6	35	292	21	7.19
7	35	371	16	4.31
8	27	502	10	1.99
9	10	245	5	2.04
10	10	217	3	1.38
11	10	164	1	0.61
12	10	169	6	3.55
13	10	124	1	0.71
14	10	181	1	0.55
15	10	108	1	0.92
Natural feeding stuff and pure vitamins				
16	217	3129	51	1.61

TABLE 5. *Blind Young Weaned from Females Receiving Various Combinations of Control and Irradiated Green Beans (GB) and Chicken (CK)*

Group No.	Treatment*		Females† Observed, No.	Young Weaned		
	GB	CK		Total No.	Blind	
					No.	%
1	0	0	54	345	2	0.58
2	0	×	18	129	0	0
3	0	2×	18	213	2	0.94
4	×	0	18	126	2	1.59
5	×	×	18	148	2	1.35
6	×	2×	18	95	0	0
7	2×	0	18	79	2	2.53
8	2×	×	18	198	0	0
9	2×	2×	18	95	1	1.05

* 0 = No treatment; × = 2.79 megarad; 2× = 5.58 megarad.
† Total females in parent, F_1 and F_2 generations.

Normal variation in the incidence of blindness. Starting in January, 1957, a record was kept of every blind young regardless of the diet the mother received in order to obtain data on the normal variation in the incidence of blindness in the colony. Reproduction data were available for 16 diets that were not used in the study on the nutritive value of irradiated diets. The number of young weaned and the number and percentage of blind young for each diet are given in table 4. The composition of the synthetic diets in table 4 was similar to that used in the irradiated food study. The main difference was in the level of protein, fat or vitamins such as niacin, inositol or biotin. The percentage of blind young ranged from 0.55 to 8.59, which was a larger variation than that obtained from mothers receiving the irradiated or control diets. Blind young occurred also in groups receiving a diet composed of natural feeding stuffs and supplemented with pure vitamins. The percentage 1.61 was as high as that obtained in the repeat trials with irradiated foods.

Blind young from green bean and chicken diets. Blind young were weaned also from mothers receiving various combinations of irradiated and control green beans and chicken. These data, which include the total number of young weaned from mothers in the parent, F_1 and F_2 generations, are summarized in table 5. The incidence of blind young was relatively low in all groups receiving the green bean-chicken diets and there were no blind young in three groups (2, 6 and 8) even though one or both foods had been irradiated. If the blindness had been caused by the irradiated food, it should have developed in young from these three groups just as readily as it did in other groups receiving the irradiated foods. Blindness did not develop in some groups receiving irradiated foods and a relatively high incidence developed in other groups which received foods that were not irradiated. If the blindness had been caused or increased by the irradiated diet, the incidence of blindness would have been consistently higher each time the diet contained an irradiated food. Therefore, it is concluded that the irradiated diet did not produce blindness or increase its incidence in weanling rats.

SUMMARY

In one series of tests, four generations of rats received an irradiated synthetic diet. Growth and reproduction performance of the animals receiving the irradiated diet were essentially equal to those of animals receiving the nonirradiated or control diet.

In a second series of tests, four generations of rats received nine different combinations of control and irradiated green beans and boned chicken. Each food was irradiated at dose level of zero, 2.79, and 5.58 megarads.

No significant differences in gain in weight at 12 weeks occurred in the parent, F_1, and F_2 generations for any combination of the irradiated and control foods. Likewise, irradiation of the food did not effect the hematologic or histopathologic picture or the weights of heart, lungs, liver, spleen, kidneys, or testes.

A few blind young were weaned from mothers receiving both the irradiated and control foods. Blindness occurred also in groups of young from mothers receiving synthetic diets which had not been irradiated. In some cases, the incidence of blindness was higher in the young from these diets than it was from the irradiated. The overall data show that the blindness which occurred was not due to the irradiated food.

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Effect of irradiated meat upon growth and reproduction of dogs[1]

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This report summarizes the results of a series of studies to determine the effect of feeding irradiated meats on the growth rate, health and reproductive performance of dogs over a 2 or 3 year period. The meats studied were beef, pork, tuna fish and chicken stew.

EXPERIMENTAL

Purebred beagle pups produced in our kennel were used because of their uniformity and because we have extensive experience and normal values on this breed.

The dogs used were given distemper and hepatitis immunization shortly after weaning. They were transferred to individual cages having expanded metal bottoms. The kennels were cleaned daily and the expanded metal bottoms and partitions were washed with a coarse brush which was dipped in a hot, dilute solution of cresol antiseptic. As far as possible dogs were confined to the same pen and seldom moved. During the winter the dogs were housed in a heated kennel, but in the summer months (Mar. 1–Nov. 15) they were kept out of doors in movable pens with elevated expanded metal bottoms.

Frozen, raw beef was beta irradiated by the Van de Graaf at the High Voltage Engineering Corporation of Burlington and Cambridge, Massachusetts. Levels of 2.79 and 5.58 million rads were used. Before irradiation, this ground beef (20% fat) was carefully inspected and packaged by the Cornell Meat Department. The meat was packaged in small units that weighed approximately 5 ounces each. These were sealed in moisture proof cellophane on special backing boards and the whole package did not exceed a half inch in thickness. After being sealed, the meat was immediately frozen and so held through irradiation and until it was used.

The pork, tuna fish and chicken stew were gamma irradiated by and according to army specifications. While the control pork, tuna fish and chicken stew were held frozen until use, their irradiated (2.79 and 5.58 megarads) counterparts were maintained (at room temperature) for 3 to 6 months before feeding. The pork used in these studies was thoroughly cooked before it was fed to the dogs.

Approximately half of the calories in the diet fed were furnished by a commercial dry dog feed and the other half by the meat being studied. The meats made up 35% of the dry matter of the diets and the commercial dog feed 65%. The dry feed mixture contained cooked, kibbled corn 39.7%, alfalfa meal 1.5, meat scrap 18, fish meal 1.5, soybean meal 8.5, tomato pomace 2.0, wheat flakes 5, tallow 5, wheat germ 8.5, dry skim milk 4, ground cheese 1.5, salt 0.2, dicalcium phosphate 0.75, dry brewers yeast 2, irradiated yeast 0.1, cod-liver oil 0.75, and liver meal 1. The guaranteed proximate analysis was protein 25%, fat 8 and crude fiber 4. The carotene and vitamin A content of the ration was 810 and 900 U.S.P. U per pound, respectively. Wheat germ, based on published values for vitamin E content, should have

[1] The experiments reported here were undertaken under Contract No. DA-49-007-MD-600 with the Office of The Surgeon General. The opinions expressed are those of the authors' and are not necessarily endorsed by the Department of the Army.

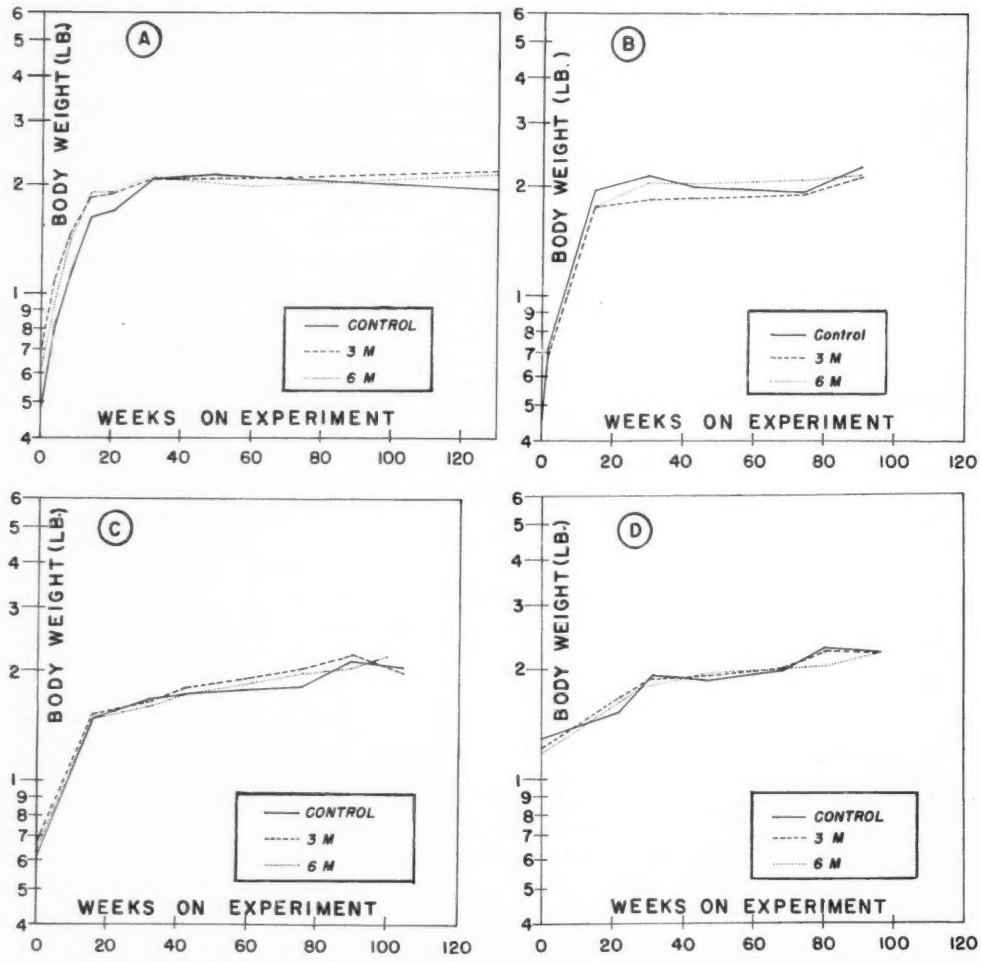

FIG. 1. Growth curves of dogs fed irradiated meats. *A:* beef; *B:* pork; *C:* chicken stew; *D:* tuna fish.

supplied approximately 5.6 U.S.P. U of alpha tocopherol per pound of meal.

Mixtures similar to this meal have been fed in our kennels for 24 years in feeding trials with dogs and have been used as a stock diet for rats and hamsters during the past 15 years. Tests with rats have indicated that this mixture can be diluted with an equal weight of sucrose and still supply adequate nutrients throughout the whole life span.

For the test on irradiated beef 18 registered female beagles, approximately 6 weeks of age, were divided into three groups of six each. For pork, 12 dogs were assigned into three groups of four each (2 males and 2 females). The chicken stew and tuna fish studies used 14 beagle puppies, three females and two males on each of the irradiated products. Two females and two males served as controls on each diet.

The dogs were fed twice daily until they were approximately 22 weeks old and after that once a day for the duration of the experiment. All the beef (irradiated and control) and just the control pork, tuna fish, and chicken stew were thawed over night and mixed with the commercial meal immediately before feeding. In the case of puppies, the meal was moistened to facilitate mixing with the meat.

Vitamins A, E and K[2] along with 3 mg of potassium iodide and 1 tablespoonful of cod-liver oil[3] were administered monthly. The A, E and K vitamin mixture was administered orally while the cod-liver oil was mixed with the ration. Six months after the experiment started, 5 gm of brewers yeast were added to the diet daily and 0.5 lb. of raw sheep or beef liver was fed weekly.

Red, white and differential cell counts, and hemoglobin values were determined on the blood of dogs at intervals of 2 or 3 months throughout the experiment. Fecal examinations for parasites were made approxi-

[2] Vitamin supplement supplied vitamin A—2500 IU, vitamin E—2.0 IU, vitamin K—16 mg per dose (1 ml).

[3] The tablespoonful (30 gm) furnished 60,000 IU of vitamin A and 6,000 IU of vitamin D.

TABLE 1. *Blood Data—90 Weeks on Experiment*

	Beef*	Pork†	Chicken Stew†	Tuna Fish†
		Control		
Hb, gm/100 ml	15.2	16.1	16.0	15.0
RBC, million	6.97	5.69	6.80	6.56
WBC, 1000	22.2	14.4	12.7	13.5
Basophils %	0.2	0.0	0.5	0.0
Eosinophils %	3.0	0.0	3.3	0.8
Lymphocytes %	32	32	37	38
Seg. neutrophils %	58	60	53	51
Band. neutrophils %	3.5	4.0	5.0	8.0
Monocytes	3.3	2.0	2.0	3.0
		5.58 Megarads		
Hb, gm/100 ml	14.5	15.9	15.4	15.2
RBC, million	6.76	6.85	6.87	6.54
WBC, 1000	20.8	14.0	13.8	12.9
Basophils %	0.2	0.5	0.0	0.4
Eosinophils %	1.0	0.8	0.6	3.0
Lymphocytes %	30	33	31	26
Seg. neutrophils %	62	57	60	60
Band. neutrophils %	4.6	7.0	6.0	6.0
Monocytes	2.0	2.8	3.0	3.0

* Av. 6 dogs. † Av. 4 dogs.

mately every 6 weeks. There was no interface with the diet or feeding procedures when anthelmintic treatment was necessary. X-rays of the radius-ulna were done when blood samples were taken. Body weights were recorded weekly during the early part of the experiments and monthly after mature body weights were attained.

The female dogs used in these studies were observed daily for estral signs and bred at each estrus. Proven stud dogs used in mating the females were fed stock diets and were from the regular kennel population.

RESULTS

Growth. Growth curves of the dogs on four diets are shown in figure 1. Two dogs on the chicken stew diet died early in the study from distemper but were immediately replaced. The weights through the first year reflect normal growth. The growth curves between the control and irradiated meat groups did not reveal any significant differences. The fluctuations in weights after 1 year were mostly due to pregnancies. Long bone measurements from the x-rays supported the body weight data.

Hematology. Blood counts of the dogs at 90 weeks are given in table 1. An analysis of variance did not reveal any significant difference in the blood data on the dogs on different levels of radiation within any time interval. As might be expected, the red blood counts and hemoglobin increased steadily with the age of the animals. The white blood counts decreased somewhat irregularly with increasing age, as expected. The slightly higher white blood cell count for the beef diet is unexplainable but it occurred on the control and irradiated diets alike. There was no evidence of parasitism or secondary infection. The pattern of the white cell differential counts was normal.

Reproduction. Data on the reproductive performance of the female dogs fed the irradiated and control meat diets are given in table 2.

On the basis of the numbers of conceptions and pups produced, and considering the small numbers of females

TABLE 2. *Reproduction*

Dog No.	No. of Times Bred	No. of Conceptions	No. of Pups Whelped	No. of Pups Weaned
Beef—0 diet				
53	3	3	12	4
50	3	3	14	13
54	3	3	22	17
69	5	3	10	9
*43**	1	1	0	0
*63**	2	1	7	1
Total	17	14	65	44
Beef—2.79 diet				
40	4	0	0	0
41	3	2	11	9
49	3	0	0	0
55	3	3	22	17
66	1	1	4	3
67	3	3	14	3
Total	17	9	51	32
Beef—5.58 diet				
42	4	3	10	5
44	3	3	7	7
48	1	0	0	0
52	4	2	12	6
62	4	1	7	0
68	2	2	8	4
Total	18	11	44	22
Pork—0 diet				
56	2	2	11	11
57	3	2	11	10
Total	5	4	22	21
Pork—2.79 diet				
55	3	1	2	2
61†	0	0	0	0
Total	3	1	2	2
Pork—5.58 diet				
58	1	1	8	6
63	2	2	11	6
Total	3	3	19	12
Chicken—0 diet				
12†	0	0	0	0
35	3	3	20	19
Total	3	3	20	19
Chicken—2.79 diet				
21	1	1	0	0
16	2	2	10	10
36	3	2	10	8
Total	6	5	20	18
Chicken 5.58 diet				
14	2	2	13	7
15	4	4	22	20
36	2	1	4	4
Total	8	7	39	31

* Died after approximately 70 weeks on experiment during whelping.

TABLE 2. *Continued*

Dog No.	No. of Times Bred	No. of Conceptions	No. of Pups Whelped	No. of Pups Weaned
Tuna—0 diet				
3	2	1	8	7
4†	0	0	0	0
Total	2	1	8	7
Tuna—2.79 diet				
12	1	1	3	2
40	1	1	8	8
41	2	2	15	15
Total	4	4	26	25
Tuna—5.58 diet				
75	1	1	3	2
38	1	1	9	8
42	1	1	5	5
Total	3	3	17	15

† Never evidenced estrus.

studied, there was no consistent evidence that irradiation impaired reproductive performance on the pork, chicken stew, and tuna fish diets.

From the data given in table 2, it appears there may have been factors present or absent in the irradiated beef that interfered with conception. There is no correlation between the amount of irradiation the beef received and the conceptual difficulties. On the beef experiment the litters were somewhat smaller and mortality of pups slightly heavier than for our kennel controls.

The post mortem and histopathological studies have not shown anything which would explain the impaired reproductive performance. Post-mortem data on puppies dying during the neonatal periods are not available.

DISCUSSION

The hematological and growth data do not indicate any detrimental effects from the feeding of irradiated foods. The lowered conception rates of the females on irradiated beef were the only suggestions of a possible harmful effect of irradiation in this experiment.

Reber (personal communication) and Blood (personal communication) in similar studies with dogs fed irradiated beef did not find any harmful effects during a 2-year period. Reber, with six female dogs, reported that each of 12 breedings resulted in conceptions. Blood observed 9 pregnancies out of 18 in his dogs, but believed this unsatisfactory conception rate was due to improper housing of the dogs.

Anderson *et al.* (1, 2) and Elvehjem *et al.* (3) reported that dogs fed a diet of mineralized evaporated milk developed a vitamin E deficiency evidenced by muscular paralysis, hemorrhagic conditions, and poor development of first generation pups. Nowhere in the experiments of Anderson and Elvehjem were there reported conceptual difficulties similar to the ones we observed.

Although a vitamin E requirement for dogs in terms of reproduction has not been definitely established, the National Research Council report (5) allows for maintenance 1 mg of vitamin E per pound of body weight.

The dogs on irradiated beef never received less than 4 ounces of commercial meal per day. It was estimated that these 4 ounces would contribute 1.4 IU of vitamin E and 428 IU of vitamin A. In addition, the monthly supplements of a vitamin mixture and cod-liver oil contributed a total of 62,500 IU of vitamin A and 2.0 IU of vitamin E. Taking the National Research Council's allowances of 1.0 IU of vitamin E and 45 IU of vitamin A per pound of body weight as a desirable intake, it seems possible that these vitamins were present in submarginal amounts in the test diets.

Reber administered the vitamins to his dogs in capsule form once weekly at the following levels: 30 mg, 5000 IU, and 500 IU of vitamins E, A, and D per 4 lb. of body weight, respectively. Blood supplied vitamins in the form of cod-liver oil and wheat germ oil. These were administered on alternate days and were placed directly on the food twice weekly.

The fact that in our experiments the cod-liver oil was mixed with the meal and fed the same day the vitamin supplement was administered might possibly have favored vitamin E destruction also.

The existence of oxidizing conditions under the influence of irradiation has been demonstrated repeatedly. Polling *et al.* (4) encountered a partial vitamin E deficiency in rats while feeding irradiated beef. Once these initial marginal deficiencies were corrected, no major differences existed between the control and experimental groups. This peroxide-vitamin E destruction might well have been the case in our beef experiment. Analyses of different meat samples indicated definite elevation in peroxide values of irradiated over control beef. It is also quite probable that peroxide values could have increased during the storage periods. Wherever the destruction might have occurred, it appears the irradiated beef component of the diet might have been submarginal enough in vitamin E to have caused reproductive impairment.

It is planned to repeat this experiment with a greater number of dogs and to give closer attention to the intakes of the fat soluble vitamins.

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Heart lesions in mice induced by feeding irradiated foods[1]

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IN A STUDY DESIGNED SPECIFICALLY to test the possible carcinogenicity of irradiated foods, a diet composed of treated pork loin, evaporated milk, blanched carrots, chicken and white potatoes was fed to mice of the Strong A and Cb strains. It soon became apparent that some Cb mice ingesting irradiated foods acquired, after a time, a severe dyspnea caused by dilatation of the left auricle of the heart and a secondary compression and displacement of the remaining thoracic viscera. The pathology of the lesion and attempts made to establish the etiology of the disease are discussed in this report.

METHODS

The following diet was employed, the calculation being based on each food providing 20% of the dry solids: pork loin, 8.8%; chicken, 6.0%; milk (evap.), 19.3%; white potatoes, 22.9%; carrots (blanched), 43.0%.

Expressed on a percentage basis of total dry weight, the diet contained: protein, 29.7%; fat, 24.3%; carbohydrate, 40.5%.

All foods were irradiated at the Gamma Facility, MTR, Phillips Petroleum Company, Idaho Falls, Idaho. The radiation levels employed were 5.58 megarad for all foods except potatoes, the latter receiving not less than 10,000 and not more than 12,000 rads.

All irradiated foods, except potatoes, were stored in an air conditioned room maintained at 76 ± 1°F for the 3 or more months before used. Each food was cooked separately in a pressure cooker at 15 pounds pressure for 20 to 30 minutes before it was compounded. The meat products were ground in a Hobart commercial food grinder. All foods were finally mixed and homogenized in a Waring Blendor.

Nonirradiated fresh frozen foods were used for a control. They were stored at −10°F until used and irradiated as well as nonirradiated potatoes were kept at temperatures ranging from 40 to 50°F.

A complete vitamin supplement (Nutritional Biochemicals Vitamin Diet Fortification Mixture) was added daily and only to that amount of diet required to fill the food dishes. Two and one half g of the above mixture and 1.5 g of Nutritional Biochemicals' salt mixture were added to 100 g of diet.

A total of 377 weanling animals (equal number of males and females) of the Cb strain were placed on the irradiated diet and a group of 370 littermate controls received an identical, nonirradiated ration.

Strong A strain animals were treated similarly, 400 and 388 animals on the irradiated and the nonirradiated diets, respectively.

RESULTS

Production of the lesion. Seven months after the initiation of feeding studies one Cb mouse on the irradiated diet died because of a ruptured left auricle. Since then, a total of 66 animals (37 males and 29 females) have died or been killed as a result of left auricular dilatation. This, at the present time, represents approximately 70% of all animals that are now dead (17.5% of total group). The monthly distribution of deaths due to the lesions is recorded in table 1.

A total of nine mice of the Strong A strain receiving irradiated food have died with lesions identical to those observed in strain Cb animals.

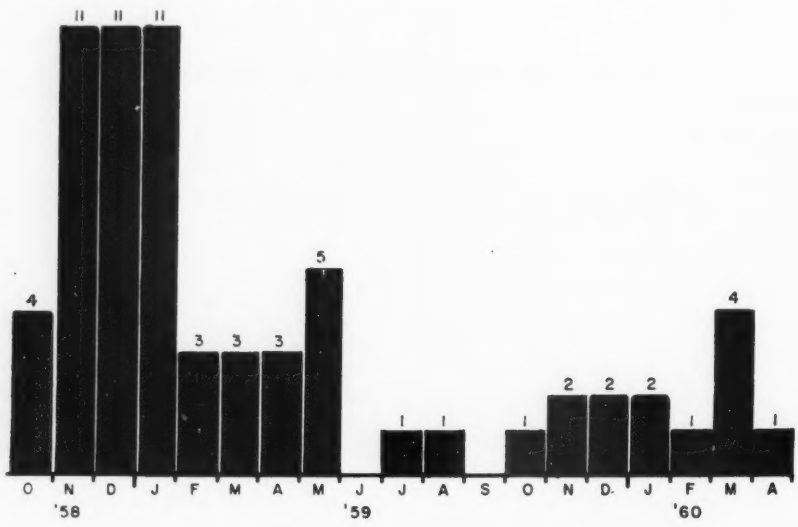
All animals exhibiting severe respiratory distress to the extent of being unable to move about in the cage were killed. While every case of auricular dilatation was recognizable clinically and consequently carefully watched, death did occur in some suddenly and unexpectedly because of rupture of the auricle. The condition often evolved without any overt signs of disease such as weight loss and anorexia. In cases where death did not intervene, a chronic state was reached in which the animals would lose much weight and become anemic.

The rapidity with which the lesion developed is indicated by the fact that one animal presented a clinical picture of auricular dilatation after having been on the diet only 48 days. The actual presence of the lesion was confirmed by autopsy. The average latent period for the development of heart disease was 218 days in females and 213 days in males, with a range of 48 to 616 days.

Description of the lesion. Autopsy always revealed a greatly distended left auricle which displaced the heart and lungs toward the right posterior thoracic wall (see fig. 1). Variable degrees of hemothorax were observed routinely. The auricular wall had a mottled appearance

[1] The experiments reported here were undertaken under Contract No. DA-49-007-MD-794 with the Office of The Surgeon General. The opinions expressed are those of the author's and are not necessarily endorsed by the Department of the Army.

TABLE 1. *Heart Lesions in Cb Mice: Monthly Distribution of Deaths*



and frequently a large solid thrombus occupied as much as 25% of the dilated auricle. There were no other gross pathologic findings.

Microscopic examination of tissues established with reasonable certainty that the involvement of the heart was limited to the left auricle. The pectinate musculature was overdistended and often fragmented. Focal areas of myocardial necrosis with heavy infiltration of leukocytes were seen in the auricular wall proper and in the trabeculae (fig. 2). Concentrations of leukocytes were prominent along the endocardium in areas with no demonstrable degenerative changes of the myocardium (fig. 3). A concentric arrangement of alternating layers of fibrin, red blood cells and trapped polymorphonuclear cells was the characteristic feature of mural thrombi (fig. 4). Bacterial stains failed to demonstrate the presence of any microorganisms. No cultures for bacterial contamination were made.

Etiology of the lesion. In view of numerous reports on extensive vitamin destruction during and following irradiation of foods, it was speculated at first that auricular damage might be one expression of some avitaminosis in the Cb strain of mice fed the irradiated diet. With the hope that a vitamin-free diet might augment such an effect, 76 males and females were placed on an irradiated diet devoid of vitamin supplement. All of these animals are still living and in excellent condition (315 to 440 days later), as are also 36 control males and females on a nonirradiated, nonsupplemented diet.

An attempt to reproduce the original findings was made by placing 142 additional animals on an irradiated fully supplemented diet. No heart lesions have as yet been discovered in this group, 240 to 360 days later.

The feeding of uncooked, irradiated evaporated milk only and vitamin supplement to 27 Cb strain males and females produced auricular dilatation in 66% (18 animals, average latent period: 75 days; range: 55 to 141 days).

FIG. 1. Left auricular dilatation with displacement of remaining heart toward right posterior thoracic wall.

In an effort to determine whether cooking the evaporated milk prior to feeding would affect the incidence of

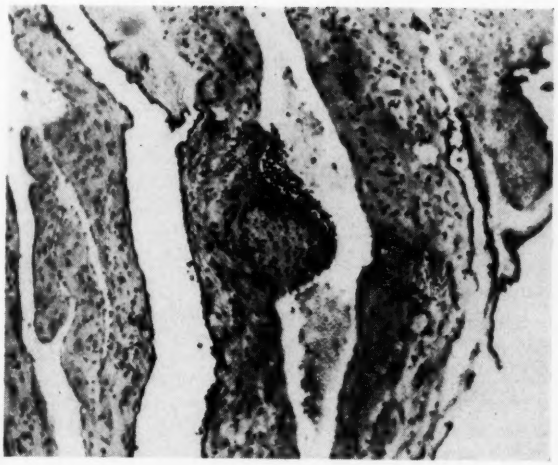


FIG. 2. Early formation of thrombus in auricular wall. Note the heavy accumulation of leukocytes. 105×.

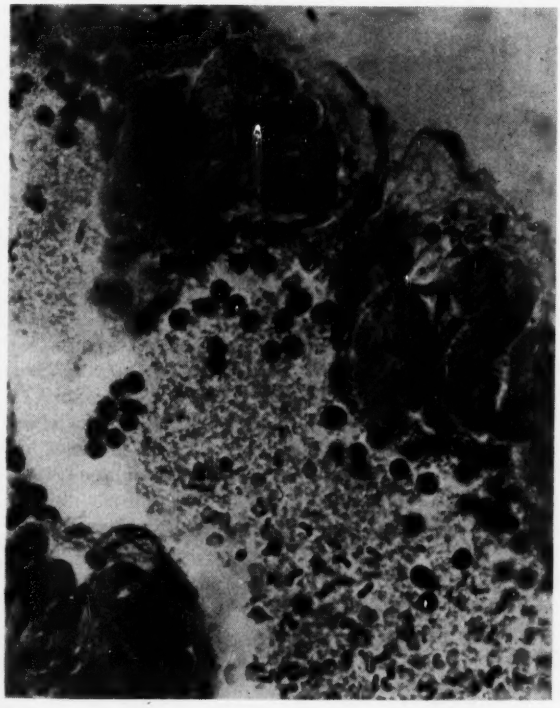


FIG. 3. Leukocytes 'sticking' to endocardium in an area where no myocardial damage was observed. 445×.

heart lesions, groups of 30 animals each were placed on the following diets: irradiated uncooked milk; irradiated cooked milk; nonirradiated uncooked milk and nonirradiated cooked milk. All groups received vitamin supplement. To obviate any effect of a possible qualitative difference in the milk of different cans, the contents of each can was divided in two; one half was fed uncooked and the other half cooked.

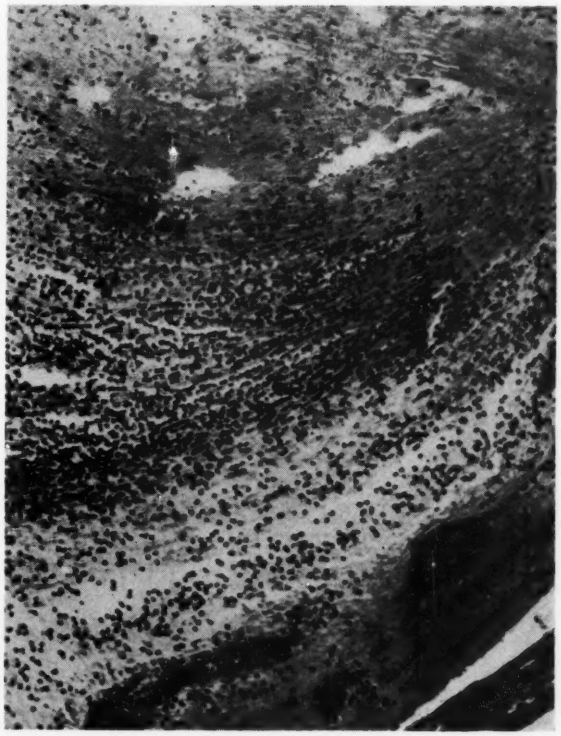


FIG. 4. Section of large thrombus in left auricle demonstrating the concentric arrangement of alternate layers of leukocytes, red blood cells and fibrin. 107×.

Twenty-five animals (83%) eating irradiated cooked milk died or were killed because of heart lesions occurring within 85 days after initiation of feeding (average: 66 days; range: 48 to 85 days). Only four lesions have been observed during the same time period in the group fed irradiated uncooked milk. No lesions developed in the controls.

DISCUSSION

An inspection of the data in table 1 reveals that the largest number of animals died or was killed in October November, December, 1958 and January, 1959. In view of the unsuccessful efforts made to induce heart lesions when feeding a diet identical in composition at a later time, it is conceivable that the etiologic agent (or agents) was present in a greater concentration in the food consumed during the fall of 1958 than during subsequent months. If this were the case, all animals that have died since January or February 1959 may have sustained, during the fall of 1958, mild cardiac injury which became clinically recognizable only much later because of 'subthreshold' levels of the responsible dietary component.

Only the irradiated evaporated milk has so far been directly implicated as a food capable of inducing heart lesions. While the feeding of irradiated uncooked milk yielded a high percentage of animals with auricular dilatation, the potentiating effect of cooking the milk prior to feeding is strongly indicated.

Nine animals of the Strong A strain developed heart lesions that were identical in every respect to those observed in Cb mice. The much higher incidence of auricular damage in strain Cb animals suggests that genetic predisposition is an important factor. This may explain why similar lesions have not been seen by other investigators who have been feeding diets containing irradiated evaporated milk.

SUMMARY

A diet composed of irradiated pork loin, chicken, evaporated milk, blanched carrots and white potatoes was fed to mice of the Strong A and Cb strains.

In the Cb group of mice, 17.5% of all animals on the irradiated diet died or were killed because of rupture and/or dilatation of the left auricle of the heart. Only 2% of the Strong A mice developed similar lesions. No heart lesions have ever been observed in litter mate control mice of both strains receiving an identically composed nonirradiated diet.

The feeding of irradiated, evaporated milk only to animals of the Cb strain resulted in the death of 60–80% of the mice due to heart lesions.

Carbonyl compounds as inhibitors of lipid metabolism and their significance in irradiated foods[1]

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EXPOSURE OF ANIMAL TISSUES to sterilizing doses of ionizing radiation results in the formation of appreciable amounts of fat soluble carbonyl compounds. A number of investigations have been concerned with the contribution of these carbonyl compounds to the characteristic flavor and odor of irradiated meats (2, 5, 6, 12). The investigations to be described here were primarily concerned with evaluating the physiological significance of the ingestion of such compounds as constituents of irradiated meats.

CARBONYL COMPOUNDS OF IRRADIATED MEATS

A joint effort between our laboratory and Drs. Tappel and Groninger (8) of the University of California was directed at determining the nature and the amounts of aliphatic carbonyl compounds to be expected in certain irradiated meats. The tissues studied were finely ground, packed under nitrogen, and subjected to radiation from spent fuel rods. The exposure of pork, beef, and chicken muscle, and of beef liver to doses of 4.6 million rad produced 2 to 3 fold increases in carbonyl compounds extractable from the tissues with iso-octane. Radiation doses of 9.3 million rad produced increases of 5 to 6 fold over the controls. The irradiation of tuna muscle produced, in contrast, a decrease in iso-octane-soluble carbonyl compounds.

These studies revealed further that the presence of the carbonyl compounds in irradiated meats was not merely transient. Approximately 50% of these compounds survived storage of the tissue for 4 months. The carbonyls were resistant also to removal or destruction by cooking (8).

Using chromatographic and spectrophotometric techniques developed expressly for this purpose (7), it was possible to obtain a great deal of information about the identity of the carbonyl compounds in the iso-octane extracts of the irradiated meats. These compounds are thought to originate by direct and by water-mediated effects of ionizing radiation on unsaturated bonds in lipid molecules, specifically the unsaturated ether moieties of plasmalogens and the points of unsaturation in oleic, linolenic, and arachidonic acids. The unsaturated ether moiety of plasmalogens would be ex-

[1] The endeavors reported here were supported in part by Contract DA-49-007-MD-631 from the Office of The Surgeon General, U.S. Army. The opinions expressed are those of the author and are not necessarily endorsed by the Department of the Army.

pected to give rise to carbonyl compounds with 15 or more carbon atoms in their chains, while the oxidative disruption of the unsaturated fatty acids would produce carbonyl compounds of from three to about ten carbon atoms.

The data obtained from the study of the beef, pork, and chicken tissue (8), together with some earlier work from our laboratory,[2] permitted the following generalizations. The unsaturated ether moieties of plasmalogens are relatively more susceptible to radiation, as indicated by the appearance of carbonyl compounds of 14 or more carbon atoms with doses of from 1 to 5 million rad. Higher doses did not seem to lead to further increases in these long chain compounds. Oxidation of unsaturated fatty acid chains became apparent at doses between 2 and 5 million rad, and was far more pronounced at 10 million rad, the highest radiation dose employed in these studies. Evidence was found for the existence of nearly every predicted carbonyl product of the oxidative disruption of the unsaturated fatty acids common to the tissues studied.

The information obtained in these studies facilitated subsequent experiments on the physiological significance of carbonyl compounds of irradiated meats by enabling us to study the physiological effects of representative synthetic compounds rather than of irradiated foods themselves.

DIGESTION AND ABSORPTION OF LIPIDS

Schreiber and Nasset (11) have reported that the irradiation of lard at 6 million rep substantially reduced its rate of removal from the gastrointestinal tract of dogs.

Dr. Gambal in our laboratory was able to demonstrate an adverse effect of aliphatic carbonyl compounds like those encountered in irradiated foods on the digestibility of corn oil. In these experiments, corn oil containing a trace of palmitic-1-C^{14} acid was administered orally to rats. The animals were sacrificed 3 hours later, and the contents of the stomach and intestinal lumens recovered for measurement of the remaining isotope. From the data of table 1 it is apparent that when 20% of the corn oil was replaced by decyl aldehyde, a substantial retardation of fat absorption occurred. Approximately twice as much of the isotope was recovered from the gastrointestinal tract when the aldehyde was present. The magnitude of this inhibition compared favorably with that observed in the experiments with irradiated lard (11).

In an attempt to ascertain the mechanism whereby carbonyl compounds exerted their effect in the above experiment we attempted to consider lipid digestion and lipid absorption as separate processes. Digestion was studied *in vitro* using purified hog pancreatic lipase and synthetic emulsions of lipids in polyvinyl alcohol (4). It was immediately apparent that the action of pancreatic lipase was retarded by the presence of decyl aldehyde (table 2). Further studies have demonstrated that fat-soluble aliphatic carbonyl compounds in general are inhibitors of pancreatic lipase when studied *in vitro*. A comparison of *n*-heptanal, *n*-decanal, *n*-dodecanal, *n*-heptan-4-one, and *n*-nonan-2-one at equimolar concentrations in corn oil emulsions revealed that they were essentially equal in effectiveness as inhibitors of lipase (unpublished data).

TABLE 1. *Aldehyde Inhibition of Fat Digestion and Absorption*

Administered	Total Counts ($\times 10^{-6}$) Recovered from Gastrointestinal Tract After 3 Hr
Corn oil	6.3
	9.6
	8.5
Corn oil + 20% decyl aldehyde	17.7
	14.7
	18.8

TABLE 2. *Inhibition of Lipase by Decyl Aldehyde*

Decyl Aldehyde in Corn Oil,* %	Inhibition,† %
0	0
3	10
5	15
10	38
20	55

* 9 ml of corn oil were homogenized with 100 ml of 1% polyvinyl alcohol preadjusted to pH 8.8. Decyl aldehyde, where included, was added to the corn oil prior to homogenization to achieve the concentrations indicated. † The rate of enzymatic hydrolysis was determined by titration of the fatty acids released during incubation of 5 ml of an enzyme suspension with 10 ml of substrate emulsion at 37°C for 1 hr.

Carbonyl compounds like those employed in our experiments are surface active materials. Pancreatic lipase works at the lipid-water interface and is sensitive to the degree of dispersion of the substrate emulsion (10). Therefore, it was suspected that the carbonyl compounds in our experiments were reducing the degree of dispersion of the corn oil in the synthetic emulsions, thereby producing a reduction in lipid surface and an effectively lower substrate concentration for the enzyme. However, turbidimetric measurements revealed that light scattering by the emulsions was actually increased by the presence of the carbonyl compounds, suggesting, contrary to our expectations, that the droplet size in the emulsion was in fact reduced by the presence of carbonyl compounds. This indication was confirmed when microscopic counting of the lipid droplets in a bacterial counting chamber demonstrated a direct correlation between the number of droplets per unit volume of emulsion and the concentration of aldehyde in the emulsified oil.

From measurements of this kind it was demonstrated that as the concentration of aldehyde in the corn oil is increased, the total lipid surface of the emulsion is actually increased, as demonstrated in table 3. This might be expected to produce an effectively higher concentration of substrate and perhaps a stimulation of lipase action. However, comparison of tables 2 and 3 reveals an inverse correlation between total lipid surface area and susceptibility to lipase.

[2] K. J. Monty and P. B. Pearson, Progress reports VI and VII to the Office of The Surgeon General.

XUM

TABLE 3. *Effect of Decyl Aldehyde on Emulsion Surface Area*

Aldehyde Concentration in Corn Oil, %	Increase in Total Emulsion Surface Area, %*
1	1
10	11
20	23

* Surface area was calculated using the formula $S = \left(\frac{12\pi c^2}{a^2}\right)^{1/3} n^{-2/3}$ where S = average surface area per emulsion droplet, c = weight of lipid per volume of emulsion, a = specific gravity of emulsion droplets, and n = number of droplets per volume of emulsion.

Pancreatic lipase has been reported to act only upon emulsified molecules (9). Both substrates and inhibitors of the enzymes apparently must be in emulsified form to be effective (3). The enzyme is seemingly unaware of lipid molecules when they exist in molecular dispersion. For these reasons, the inhibitory action of carbonyl compounds upon pancreatic lipase is probably explainable in terms of an alteration of some property or properties of the surface of the emulsion droplets. In other words, the carbonyl compounds alter the nature of the substrate rather than affect the enzyme itself. In this connection it is important that the effects of the carbonyl compounds are fully reversible. Lipase is not denatured by exposure to carbonyl-containing emulsions.

Bangham and Dawson (1) have reported that the magnitude of the electrophoretic charge on the surface of the substrate emulsion is important to the action of a lecithinase. Some preliminary experiments in our laboratory have indicated that the electrophoretic properties of corn oil emulsions are modified by the presence of carbonyl compounds. However, our measurements have not been precise enough to recognize a correlation between surface charge density and susceptibility of the emulsion to lipase, if such a correlation does exist. At the present moment other effects, such as surface tension and close packing of carbonyl molecules at the droplet surface, must be considered as possible explanations of the phenomenon.

The inhibitory effect of carbonyl compounds upon the action of pancreatic lipase seemingly would be adequate reason for the retarded rate of removal of carbonyl-containing lipids from the gastrointestinal tract. However, the process or processes of absorption appear to be affected as well.

In experiments similar to those described earlier, corn oil was introduced by oral intubation into the stomach of fasted rats. The corn oil contained a trace of palmitic-1-C^{14} acid of high specific activity. Three hours after the administration of lipid, the animals were sacrificed and the lipoidal contents of the stomach and intestinal lumens recovered.

The results of these experiments are summarized in table 4. The data are expressed as specific activity of C^{14} in the recovered lipid, and are averages obtained from several animals employed in the experiments.

When corn oil is administered alone, it is hydrolyzed in the intestinal lumen. The free fatty acids released by the hydrolysis are to a considerable extent absorbed in preference to the lipid which has not yet been hydrolyzed. The labelled palmitic acid becomes an integral part of the free fatty acid pool, and is preferentially absorbed with it. Consequently, a drop of about 50% in the specific activity of the contents of the intestinal lumen is observed. The specific activity of the stomach contents remains essentially equal to that of the administered lipid.

When 20% of the corn oil is replaced by decyl aldehyde, the selective absorption of the labelled fatty acid is reduced. The inhibition of lipase by the aldehyde was a possible explanation of this result, since the labelled fatty acid is only a trace amount of material, and cannot be selectively absorbed until it is augmented substantially by free fatty acids released by the action of lipase.

A third group of animals received a lipid mixture wherein 5% of the corn oil was replaced by free palmitic acid. This mixture represented a partially predigested lipid. Under these conditions the radioactive palmitic acid should be selectively absorbed as part of the free fatty acid pool, and a resultant drop in the specific activity of the intestinal contents observed. However, the presence of the decyl aldehyde in the ingested mixture retarded this selective absorption.

The conclusion that carbonyl compounds affect the absorption as well as the digestion of fats seems inescapable. This situation is perhaps not surprising, since proper emulsification is a prime requisite to the absorption of lipid from the intestinal lumen. There is considerable data in the literature to indicate that lipid hydrolysis in the intestinal lumen occurs for the sole purpose of facilitating proper emulsification. Inhibition of lipid absorption in our experiments is apparently another reflection of the ability of carbonyl compounds to modify the surface properties of emulsions.

In an attempt to establish whether carbonyl com-

TABLE 4. *Aldehyde Inhibition of Fat Absorption*

Administered	Specific Activity (cpm/mg Lipid) After 3 Hr	
	Stomach	Intestine
Corn oil	2023	927
Corn oil + 20% decyl aldehyde	2092	1436
Corn oil + palmitic acid + decyl aldehyde	2400	1590

TABLE 5. *Carbonyl Content and Lipase Susceptibility of Lard and Corn Oil*

Emulsion	Total Carbonyl Content (mmoles/100 ml emulsion)	Relative Rate of Hydrolysis by Lipase
Lard	0.06	100
Irradiated lard (6MREP)	2.6	15
Corn oil	<0.02	100
Corn oil + decyl aldehyde	2.4	85
	16	54

pounds were in fact responsible for the retarded rate of absorption of irradiated lipids, we examined samples of the lard used by Schreiber and Nasset in their experiments. These samples were analyzed for total fat-soluble carbonyl compounds, and were tested for susceptibility to lipase attack in synthetic emulsions.

Irradiation of the lard at 6 million rep had produced a very great increase in carbonyl compounds, as demonstrated in table 5. Emulsions of this lard in polyvinyl alcohol were hydrolyzed by pancreatic lipase at only 15% of the rate obtained with unirradiated controls.

When corn oil was brought to the same concentration with respect to decyl aldehyde, the susceptibility to lipase action was decreased to a much lesser extent. In fact, a further 6 fold increase in decyl aldehyde failed to even approximate the retardation of lipase action noted with the irradiated lard.

Thus it would seem that irradiated lard does not contain enough carbonyl compounds to explain its resistance to lipase attack.

As has been noted by Moore,[3] the synthetic emulsions of irradiated lipids are quite unstable, showing a pronounced tendency to separate during the 40 minute incubations with lipase. Microscopic examination of these emulsions reveals droplets of large size. This is in contrast to the behavior of carbonyl-containing emulsions, which actually appear to be more stable and of finer droplet size than emulsions not containing carbonyl compounds.

We have concluded that although carbonyl compounds may contribute to the relative indigestibility of irradiated lipids, their significance is relatively minor. It seems highly probable that other surface active molecules resulting from exposure of lipid to radiation, perhaps peroxides, for example, are of far greater significance to these attributes of irradiated lipids than are the carbonyl compounds.

CHRONIC INGESTION OF CARBONYL COMPOUNDS

The central question behind these endeavors is: Do the carbonyl compounds resulting from the irradiation of lipids present any real hazard in the chronic ingestion of irradiated foods?

We have subjected rats to daily forced feeding of corn oil containing relatively enormous amounts of carbonyl compounds, approximately 50 to 100 times as much as the animals would be expected to encounter in a reasonable diet containing irradiated meats. Control animals received pure corn oil in place of the carbonyl compounds. In addition, all animals were offered a nutritionally balanced dry diet ad libitum. In experiments of 20 to 30 days duration, no gross toxic manifestations were observed in any animals; neither body weight gain nor food intake was affected by the carbonyl compounds tested; nor was the utilization of the corn oil by the animal significantly reduced. Though the carbonyl compounds appear to be absorbed by the animals, chemical analysis of the liver and brain failed to demonstrate any accumulation of the administered compounds. These chemical methods were sensitive enough to have detected less than 1 millimicromole of the ingested carbonyl compound per gram of total lipid. It would appear that the rat was capable of metabolizing the ingested carbonyl compounds adequately and rapidly enough to prevent any significant accumulation or toxic effect.

[3] R. O. Moore, Progress reports to the Office of The Surgeon General under Contract DA-49-007-MD-787.

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Vitamin K nutrition and irradiation sterilization[1]

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In December 1957, within 2 months after the initiation of a long-term study of the nutritional adequacy of irradiated beef, we discovered the widespread occurrence of deaths due to hemorrhage in male rats receiving diets containing irradiated beef. In the original diet, vitamin K had not been used because of the commonly accepted belief that vitamin K is not required in the diet of the rat. However, within a short time we had proved that vitamin K did cure or prevent this hemorrhagic syndrome. This finding has led us into a study of vitamin K nutrition and how it is related to meat irradiation, and this afternoon I would like to discuss the contribution that the irradiation sterilization program has made to our understanding of vitamin K nutrition.

The conclusions that we have drawn from the 2½ years' work are as follows:

a) The rat does require vitamin K in its diet. *b*) Irradiation does destroy (or render unavailable) most of the vitamin K normally present in beef. *c*) Coprophagy is an important factor in rat nutrition. It can vary widely between different diets and can provide part, but probably not all, of the vitamin K requirement of the animal. *d*) With coprophagy prevented, the rat is dependent upon an almost daily intake of vitamin K, and on a vitamin K-free diet develops hypothrombinemia in a few days. *e*) With coprophagy prevented the true dietary requirement for vitamin K can be quantitatively determined and is of the order of 0.1 μg of vitamin K_1 per gm diet. *f*) Irradiation does not produce any vitamin K antagonist which would increase the requirement of the animal for vitamin K. *g*) The female rat is very much less suceptible to vitamin K deficiency than is the male rat. *h*) The feeding of irradiated beef does not appear to affect the absorption of fat-soluble vitamin K. (However, this is an area which may need further investigation.) *i*) Vitamin K_1 appears to be stable when mixed in diets containing irradiated beef, while vitamin K_3 is unstable in such diets. *j*) As in the case of other vitamins, when the level of vitamin K present in the food is depressed by processing, supplementation with vitamin K-containing foods, or with vitamin K will prevent the deficiency. *k*) Not only irradiated beef but also pork, whether irradiated or not, are a poor source of vitamin K, and a survey with an adequate assay would probably show this to be true of meats in general.

It appears to us that the following areas need to be studied with regard to vitamin K nutrition and irradiation.

1) What are the pathways by which man can obtain vitamin K? Is vitamin K required in the diet, or can man obtain his complete requirements by absorption from the intestinal tract of microbially synthesized vitamin K? If the latter were true, the effects of processing on vitamin K would be of no importance in human nutrition.

2) A sensitive and specific microassay is needed to determine the vitamin K content of foods and the actual extent of vitamin K destruction with various processing methods.

3) While extensive work is being carried out on the function of vitamin K in electron transport, its role in prothrombin synthesis is still to be elucidated.

4) Further work may need to be done on the effect of irradiated meats on the absorption of both dietary vitamin K and intestinally synthesized vitamin K.

5) The rapidity of production of vitamin K deficiency in coprophagy-prevented rats fed a K-free diet is, I believe, unique in the vitamin field, in that it appears to apply to the adult as well as to the growing male rat.

6) The very marked sex difference discussed in a following paper by Dr. Mellette is also, I believe, unique in the vitamin field and worthy of continued study.

The data presented are taken largely from our own work but will include some reference to the work of others. In particular, the extensive work carried out in the Medical Nutrition Laboratories in Denver could be used equally well to substantiate the conclusions arrived at.

HEMORRHAGIC SYNDROME ON IRRADIATED BEEF

Our original beef-containing diet used for the long-term studies is given in table 1. The methods of preparing, storing, and feeding this ration have been published (11).

Table 2 lists the deaths from hemorrhage which occurred on this diet during the first 46-day period when

[1] These studies were supported in part under contract No. DA-49-007-MD-544, with the Office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.

Irradiation with gamma rays was carried out at the Atomic Energy Commission's Material Testing Reactor (MTR) at Arco, Idaho, by the Phillips Petroleum Company.

Rad is defined as 100 ergs of radiant energy absorbed per gram of the substance.

irradiated beef was fed, the first death of a male rat occurring on the 11th day. This hemorrhagic syndrome in rats consuming irradiated beef has been confirmed in over 25 subsequent experiments.

In order to test the possibility that various irradiation products in the beef might react with some of the constituents of the fat components of the original diet, these fat components were eliminated and fed orally in various forms, as indicated in table 3. The data indicate that the source of the fat-soluble vitamins is significantly unrelated to the production of the hemorrhagic deaths. Another possibility was that hemorrhages might be produced as a result of changes which occurred during storage of the irradiated beef used in the first experiments. However, the data in table 4, and subsequent data, indicate that neither storage of the beef itself nor of the beef-containing diet, affected the production of the disease.

Because of the nature of the syndrome and the fact that no vitamin K had been used in the rations, the effect of supplemental vitamin K_3 was determined, and, as can be seen from table 5, it completely prevented the hemorrhagic deaths. To study the possibility of a factor in irradiated beef which inhibits the absorption of vitamin K as it normally occurs in fats, the relative effectiveness of water-soluble and fat-soluble vitamin K_3 was studied. The data in table 6 indicate that at the levels given the fat-soluble form of vitamin K_3 in oil was active, as was the water-soluble form. Certainly there

TABLE 1. *Composition of Experimental Diets*

Constituents	%
Ground beef* (untreated or irradiated)	35
Wheat flour	35
Sucrose	19
Cod-liver oil	1.5
Wheat-germ oil	0.5
Mineral mixture 446†	4
Vitaminized cerelose‡	5

* Wet beef was used in the diet equivalent to 35% dry matter. † Mameesh and Johnson (4). ‡ Percentage composition of vitaminized cerelose: Ca pantothenate, 100 mg; nicotinic acid, 40 mg; thiamine HCl and riboflavin, 20 mg each; pyridoxine HCl, 10 mg; folic acid, 2 mg; vit. B_{12}, 0.1 mg; biotin, 0.06 mg; choline chloride, 2 gm; and cerelose, 97.8 gm.

TABLE 2. *Effect of Irradiated Beef on Growth and Mortality (a 46-day trial)*

No. and Sex of Rats	Daily Growth, gm.*	Deaths from Hemorrhage
	Nonirradiated beef	
20 males	6.9±0.13	0
20 females	4.9±0.15	0
	Irradiated beef†	
20 males	6.7‡±0.34	14
20 females	4.7±0.07	1

* Av. daily gains (with standard errors) during first 20 days of feeding. † Beef, gamma-irradiated at 5.58 megarad. ‡ Av. of 5 rats.

TABLE 3. *Effect of Oral Supplements on Survival of Male Rats Fed Irradiated Beef (a 56-day trial)*

Diet	Oral Supplement	No. of Rats	Deaths from Hemorrhage
$Beef_0$* + CLO† + WGO‡		8	1
$Beef_6$§ + CLO + WGO		8	3
$Beef_6$ + CLO	WGO	8	6
$Beef_6$ + CLO	Vitamin E + methyl linoleate	8	1
$Beef_6$ + WGO	CLO	8	4
$Beef_6$ + WGO	Vitamins A and D	8	4
$Beef_6$	Vitamins A, D, and E + methyl linoleate	8	4

* Non-irradiated beef. † Cod-liver oil. ‡ Wheat-germ oil. § Beef, gamma irradiated at 5.58 million rad.

TABLE 4. *Effect of Storage of Irradiated* Beef and of Irradiated Beef Diet on Survival of Male Rats*

Type of Beef in Diet	No. of Rats	Deaths from Hemorrhage
Stored irradiated beef†	9	9
Fresh irradiated beef†	9	2
Nonirradiated beef†	6	0
Stored irradiated beef‡	9	9

* Ground beef was gamma ray irradiated at 5.58 megarad. † Diet was mixed and stored frozen for the entire feeding period of 40 days. ‡ Diet was mixed every other day; no storage of diet.

TABLE 5. *Effect of Vitamin K_3 Oral Supplementation on Survival of Rats Fed Irradiated Beef (56-day trial)*

Type of Beef in Diet	Supplementation	No. of Rats	Deaths from Hemorrhage
	Experiment 1		
$Beef_0$*	None	10	0
$Beef_0$	1–3 μg vitamin K_3/gm of diet	4	0
$Beef_6$†	None	14	9
$Beef_6$	1–3 μg vitamin K_3/gm of diet	14	0
	Experiment 2		
$Beef_6$	3 μg vitamin K_3/day	8	0
$Beef_6$	10 μg vitamin K_3/day	8	0
$Beef_6$	30 μg vitamin K_3/day	8	0

* Nonirradiated beef. † Beef, gamma irradiated at 5.58 million rad.

TABLE 6. *Relative Effectiveness of Water Versus Fat-Soluble Vitamin K_3 in Rats Fed Irradiated Beef (three-week trial)*

Oral Supplement/Rat/Day	No. of Rats	Plasma Prothrombin Time, sec.	Deaths from Hemorrhage
None	8	24	2
1 μg vitamin K_3* in water	6	27	0
3 μg vitamin K_3 in water	6	16	0
6 μg vitamin K_3 in water	6	16	0
One drop corn oil	6	23	0
One drop corn oil + 1 μg vitamin K_3†	6	20	0
One drop corn oil + 3 μg vitamin K_3	6	15	0
One drop corn oil + 6 μg vitamin K_3	6	14	0

* Sodium salt of 2-methyl-1,4-naphthoquinone diphosphate ester. † 2-Methyl-1,4-naphthoquinone.

was no marked effect of irradiated beef on the absorption of fat-soluble vitamin K_3.

PRODUCTION OF DIETARY VITAMIN K DEFICIENCY

It has been frequently stated that the rat has no dietary requirement for vitamin K because adequate amounts are available from intestinal synthesis (12). Barnes *et al.* (1, 2) and Mameesh *et al.* (5) have found that coprophagy prevention will result in vitamin K deficiency in rats fed a vitamin K-deficient synthetic diet of the type usually used and which does not normally result in a deficiency of vitamin K when coprophagy is allowed. This demonstrated that vitamin K was microbially synthesized in the intestinal tract and was not directly absorbed, but was obtained by recycling of the feces.

Since we had apparently produced a vitamin K deficiency on the irradiated beef diet without resorting to coprophagy prevention, work was carried out to develop a synthetic type ration which would do the same thing (5). Table 7 gives the composition of the ration finally arrived at, and the data in table 8 show that this diet not only produced a severe vitamin K deficiency in a very short time (4 animals out of 6 having died in 10 days), when coprophagy was prevented, but also clearly produced a vitamin K deficiency even though coprophagy was allowed and did occur. This was, I believe, the first report of a synthetic type ration which was sufficiently low in vitamin K to produce readily a dietary vitamin K deficiency and thus to show that the rat had a dietary requirement for vitamin K which could not be completely supplied by intestinal synthesis and coprophagy.

TABLE 7. *Diet Used to Produce Vitamin K Deficiency in the Rat*

Ingredients	%
Sucrose	66.5
Soy protein*	20.0
DL-Methionine	0.5
Vitaminized cerelose†	5.0
Salts mixture No. 446‡	4.0
Wheat-germ oil	0.5
Cod-liver oil	1.5
Glycerol	2.0
Total	100.0

* Drackett assay protein. † Supplied the following in mg per kg diet: thiamine hydrochloride, 10; riboflavin, 10; calcium pantothenate, 50; pyridoxine HCl, 5; nicotinic acid, 20; folic acid, 1.0; vitamin B_{12}, 0.10; biotin, 0.10; choline chloride, 1000. ‡ Mameesh *et al.* (4).

TABLE 8. *Effect of Coprophagy Prevention and Menadione Supplementation on the Hemorrhagic Syndrome and Hypoprothrombinemia of Vitamin K Deficiency in the Rat* (10 Days)*

Diet	Oral Supplement/ Rat/Day	No. of Hemorrhagic Deaths	Plasma Prothrombin Time (Mean and Spread), Sec.	Coprophagy, %
Soy protein		1	32(14–66)	38
Soy protein	5 μg vit. K_3	0	14(13–18)	29
Soy protein†		4	92(63–120)	
Soy protein†	5 μg vit. K_3	0	15(14–16)	

* Six rats per group. † Coprophagy prevented.

TABLE 9. *Vitamin K Activity of Proteins Used in Vitamin K-Free Diets**

Dietary Protein	No. of Rats	Days on Experiment	Coprophagy	No. of Hemorrhagic Deaths	Prothrombin Time, Sec.
Labco casein	8	20	allowed (70%)	0	14
Labco casein	6	17	prevented	0	33
NBC casein	4	14	allowed	0	25
NBC casein	4	14	prevented	2	74
Drackett-soy	6	10	allowed (20%)	1	32
Drackett-soy	6	10	prevented	4	92
Beef (5.58 megarad)	6	14	allowed (1.5%)	1	77
Beef (5.58 megarad)	6	14	prevented	4	72

* All diets made up to contain approximately 20% conventional protein.

TABLE 10. *Coprophagy on Various Diets*

Diet	No. of Experiments	Mean % Coprophagy (Spread)
B_0	5	32(21–42)
B_2	3	23(9–38)
B_6	5	12(−10–+33)
Soy protein	1	38
Soy protein + 0.2% decanal	2	18(18–19)
Labco Casein	1	72
B_0 + 0.2% decanal	2	23(14–32)

Methods: All rats are fed the experimental diet ad libitum for at least 1 week. At 0 hour, tail cups are placed on all rats, and at 24 hours the rats are paired according to feed intake. One member of each pair is maintained without tail cup (coprophagy allowed); the other member is fitted with a clean cup (coprophagy prevented); and collection of feces from both members of the pair is begun simultaneously. At 40 hours (16 hours of collection) the feces are collected, dried at 80°C overnight and weighed to the nearest mg.

$$\% \text{ coprophagy} = \frac{\text{gm feces cop. prevented} - \text{gm feces cop. allowed}}{\text{gm. feces cop. prevented}} \times 100.$$

Following the development of this ration we have compared other protein sources in the same ration with and without coprophagy prevention, and these data are given in table 9. From this table it can be seen that vitamin K deficiency can be produced also on hot-alcohol-extracted (NBC) casein even when coprophagy is allowed, and on Labco casein when coprophagy is prevented. Coprophagy, however, on Labco casein is normally very high, and under these conditions a deficiency does not occur. Our belief is that the data indicate a small content of vitamin K in Labco casein, since even with coprophagy prevented it requires a considerable time for a vitamin K deficiency to develop on the Labco casein diet.

Since the extent of coprophagy appeared to be important as a part of the cause of vitamin K deficiency on vitamin K-free diets, its extent has been further examined by the method of Barnes *et al.* (3) reported in table 10. In addition, since it has been reported that irradiated meats contain significant amounts of long-chain car-

bonyl compounds, their effect on the production of the hemorrhagic syndrome and of coprophagy has also been studied, using the C^{10} and C^{18} straight-chain aldehydes.

The data in table 10 show the wide variation in coprophagy that does occur, and this variation may partially explain the extent of vitamin K deficiency which occurs on the beef irradiated at 5.58 million rad. It is interesting that the addition of decanal appeared to depress coprophagy, and it also appeared to slightly increase the incidence of vitamin K deficiency (10), as shown in table 11. Because of this effect of the carbonyls, their toxicity was tested in a direct experiment, the compounds being given by injection into the heart. As can be seen from the data in table 12, however, none of these compounds had any effect on prothrombin time; in fact, they appeared to be entirely nontoxic at these levels (V. C. Metta, unpublished data).

Because of the desire to continue the rats for long periods with coprophagy prevented, and because of the stress of doing this using the tail cup method of Barnes *et al.* (3), square-tubular wire screen cages have been developed for different size rats (Metta *et al.* unpublished data) and these are now being routinely used for this purpose. Growth on complete synthetic rations is as good in these cages as it is in the usual screen bottom cages.

Vitamin K activity of the cecal and colon contents, taken at slaughter and quick frozen, was measured by the chick assay. Expressed on the dry basis, the cecal plus colon contents were found to contain vitamin K activity equal to 8–19 micrograms vitamin K_3-sodium phosphate ester per gram. This indicates that freshly voided feces could provide considerable vitamin K (probably less than 4 to 8 micrograms per day, since fecal dry matter consumed is rarely 0.5 gm/day).

With the finding that vitamin K deficiency could readily be produced in coprophagy-prevented rats fed the soy protein diet, it then became possible to determine quantitatively for the first time the vitamin K requirements of the rat (6). The synthetic diet used for these studies is given in table 13.

TABLE 11. *Effect of Feeding Decanal to Growing Rats*

Diet	No. of Rats	Mortality Due to Hemorrhage by 75 Days
$Beef_0$	5	0
$Beef_0$ + 0.2% decanal	5	0
$Beef_0$ + 0.5% decanal	5	3
Labco casein	5	0
Labco casein + 0.5% decanal	5	2

TABLE 12. *Effect of Intracardially Administered Aldehydes*

$Beef_0$, Diet	
Dose injected	Prothrombin time, sec.
2 mg Tween 80	17
1 mg n-decylaldehyde + 2 mg Tween 80	15
2.5 mg n-decylaldehyde + 2 mg Tween 80	15
1 mg n-octylaldehyde + 2 mg Tween 80	15
1 mg $beef_6$, steam-distillate + 2 mg Tween 80	15

TABLE 13. *Basal Diet Used for the Determination of Vitamin K Requirement of the Rat*

Ingredients	Gm
Sucrose	66.9
Soy protein	20.0
DL-methionine	0.5
Triolein	3.0
Vitaminized cerelose*	5.0
Salt mixture No. 446†	4.0
Methyl linoleate urea inclusion compound (25% methyl linoleate)	0.6
α-Tocopheryl acetate (250,000 USP u/gm)	0.012
Vitamin A	0.008
Vitamin D_3 (15,000 I u/gm)	0.013

* Mameesh and Johnson (5). † Mameesh and Johnson (4).

TABLE 14. *Mean 4-Week Gains, Feed Intakes, Incidence of Lethal Hemorrhages and Plasma Prothrombin Times of Groups of Ten Noncoprophagic Rats Receiving Various Levels of Vitamin K_1*

Vitamin K_1 Administered, μg/gm diet	Average 4-Week Gain, Gm	Average 4-Week Feed, Gm	Hemorrhagic Deaths	Average Prothrombin Time, Sec.
Exper. I				
0	103±15.6*	279±19.5*	5	58.6±8.8*
0.2	169±5.3	386±5.8	0	13.2±0.2
0.4	165±4.3	380±8.6	0	13.8±0.3
0.6	162±3.3	368±6.7	0	13.9±0.2
0.8	154±4.8	359±7.8	0	14.2±0.2
1.0	159±1.8	379±4.5	0	14.0±0.1
Exper. II				
0	156	366	9	83
0.05	146±4.1	359±10.4	2	25.0±2.3
0.10	159±3.2	380±6.1	0	14.8±0.2
0.15	157±3.6	372±5.8	0	13.9±0.2
0.20	157±3.2	387±6.3	0	13.9±0.2

* Standard error of the mean.

The results at various levels of vitamin K_1 administration are given in table 14 and indicate that the requirement for vitamin K_1 of the noncoprophagic rat was between 0.1 and 0.15 μg of vitamin K_1 per gm of diet. When the reciprocal of the prothrombin time was plotted against vitamin K_1 level, the values below the requirement fitted a straight line which intersected the horizontal line of normal prothrombin time at a point corresponding to approximately 0.1 μg vitamin K_1 per gm of diet.

The vitamin K_1 requirement was then determined for male rats receiving the irradiated beef ration given in table 15. The results of this experiment, in which vitamin K_1 was given both orally and mixed with the diet, are given in table 16 and indicate that on the irradiated beef the vitamin K_1 requirement is also of the order of .1 μg per gm of diet. This supports our finding that on the irradiated beef there is little coprophagy as a source of vitamin K_1, and indicates that, for the rat at least, the irradiated beef provides very little vitamin K, yet at the

same time does not appear to contain any vitamin K antagonist.

As given in table 17, irradiated beef was compared with Drackett protein in three diets, as a source of vitamin K. From this experiment it should be possible to determine whether any antivitamin K compound was present in the irradiated beef or, also, whether the irradiated beef supplied any significant amount of vitamin K. The data from this experiment, given in table 18, indicate quite clearly the absence of any hemorrhagic compound in the irradiated beef (7) and, in fact, show that the irradiated beef does supply some vitamin K to the animals as compared to soy protein. By contrast, it has been found that the addition of sulfaquinoxaline to the vitamin K-deficient soy protein diet caused hemorrhagic death of all animals within 6 days.

In table 19 are given data on the comparative activity of vitamin K_3 and vitamin K_1 given orally each day. From this experiment it can be seen that both forms of vitamin K were equally active in preventing the vitamin K deficiency produced on the irradiated beef diet. The data in table 16 had indicated vitamin K_1 to be stable in the diet; however, when vitamin K_3 was mixed with the diet it was found to be relatively unstable. The data in table 20 show that even with 2.5 μg of vitamin K_3 per gm of the irradiated beef diet, vitamin K deficiency did appear in a 60-day experiment. Read *et al.* (unpublished data) have also found that vitamin K_3 added to a pork-containing diet to supply 20 μg per rat per day was unstable in the diet (table 21). These workers at the Medical Nutrition Laboratory not only have obtained the same results as we have on vitamin K deficiency in irradiated beef, but have extended this to pork. Some of their data given in table 21 indicate very clearly that pork, whether irradiated or nonirradiated and whether raw or cooked,

TABLE 15. *Basal Diet*

Constituents	% Gm
$Beef_6$*	35.0
Sucrose	35.0
Starch	19.0
Glyceryl trioleate†	2.0
Salts 446‡	4.0
Vitaminized cerelose§	5.0

* Wet beef was used in the diet equivalent to 35% dry matter. † Contained 0.25 gm methyl linoleate and 12 mg α-tocopheryl acetate. ‡ Mameesh and Johnson (4). § Metta *et al.* (9); also supplied 2000 USP U of vitamin A and 200 IU of vitamin D_3 per 100 gm diet.

TABLE 16. *Hemorrhagic Deaths and Plasma Prothrombin Times of Rats Fed an Irradiated Beef Diet Supplemented With Several Levels of Vitamin K_1*

Diet	Oral Supplement	No. of Rats	Plasma Prothrombin Time, Sec.	Hemorrhagic Deaths
$Beef_6$		10	32.0(14–96)	2
$Beef_6$ + 0.05 μg K_1/gm diet		5	19.4(14–28)	0
$Beef_6$ + 0.10 μg K_1/gm diet		5	14.6(13–17)	0
$Beef_6$ + 0.20 μg K_1/gm diet		5	14.2(13–16)	0
$Beef_6$ + 0.40 μg K_1/gm diet		5	14.2(13–16)	0
$Beef_6$	0.05 μg K_1/gm diet	5	19.2(17–21)	0
$Beef_6$	0.10 μg K_1/gm diet	5	16.2(15–18)	0
$Beef_6$	0.20 μg K_1/gm diet	5	15.4(14–19)	0
$Beef_6$	0.40 μg K_1/gm diet	5	14.4(13–15)	0

TABLE 17. *Effect of Increasing Levels of Irradiated Beef; Composition of Diets*

Constituents	Diet		
	A %	B %	C %
$Beef_3$*	42.0	21.0	10.5
Soy protein		17.0	25.5
Refined corn oil (Mazola)		4.0	6.0
Sucrose	18.0	18.0	18.0
Starch	29.0	29.0	29.0
Cod-liver oil	1.5	1.5	1.5
Wheat-germ oil	0.5	0.5	0.5
Salt mixture No. 446†	4.0	4.0	4.0
Vitaminized cerelose‡	5.0	5.0	5.0

* Dry substance; however, wet beef used in the diet. † Mameesh *et al.* (4). ‡ Metta *et al.* (9).

TABLE 18. *Effect of Increasing Levels of Irradiated Beef on Hemorrhagic Syndrome; Mortality Data*

Diet	No. of Rats	Hemorrhagic Deaths			
		1st week	2nd week	3rd week	Total
A*	8	0	0	2	2
B	8	0	0	6	6
C	8	1	3	2	6

* See table 17.

TABLE 19. *Comparative Potency of Menadione and Vitamin K_3 for Rats Fed Beef Diets (26 Days)*

Diet	Average Weight Grain,* Gm	Prothrombin Time, Sec.
$Beef_0$	172±6.5†	24±0.45
$Beef_6$	114±3.5	54±4.5
$Beef_6$ + 2γ K_3/rat/day‡	142±4.7	26±2.5
$Beef_6$ + 2γ K_1/rat/day‡	133±9.5	24±0.18

* 6 male rats/group. Average initial weight, 42 gm. † Standard error of the mean. ‡ Vitamin K_1 and K_3 (Menadione) in corn oil fed orally.

TABLE 20. *Destruction of Vitamin K_3 in Diet, 60-Day Feeding Experiment*

Diet	Supplemental Vitamin K_3, γ/gm	No. of Male Rats	Deaths from Hemorrhage
$Beef_0$	2.5	5	0
$Beef_3$	2.5	8	0
$Beef_6$	2.5	8	2

is a very poor source of vitamin K, and that on diets containing 35% pork on the dry basis, as had been used for beef in our previous studies, animals die with the typical symptoms of vitamin K deficiency and are normal when vitamin K is provided. Kraybill *et al.* (unpublished data) found no vitamin K deficiency in male rats fed for 12 weeks a composite diet containing meat, vegetables, fruit, eggs, milk, bread, etc., irradiated at 5.58 million rad.

In order to study the absorption of vitamin K, Read *et al.* added bile acids to the diets of rats receiving non-

TABLE 21. *Deaths of Male Rats Fed Vitamin K-Deficient Diets Containing Cooked or Raw Nonirradiated or 5.58 Megarad Irradiated Pork**

	Nonirradiated						Irradiated	
	Raw			Cooked			Cooked	
Daily vitamin K_3 supplement								
In diet (μg)	0	20	0	0	20	0	0	0
Oral (μg)	0	0	20	0	0	20	0	20
Number of rats	10	10	10	20	10	10	14	10
Deaths in:								
56 days	6	3	1†					
70 days				12	8	0	13	0
Prothrombin time, Av. in secs.	110	29	15	85	75	13	140	12

* Read *et al.* unpublished data. † Not from hemorrhage.

TABLE 22. *Deaths of Male Rats Fed Cooked and 5.58 Megarad Irradiated Beef**

Supplement	Cooked Nonirradiated		Cooked Irradiated		
Vitamin K_3, 4 γ/day					4
Bile acids, 83 mg/day		83		83	
No. of rats	15	10	10	10	10
Deaths in 70 days	3	0	10	5	0
Prothrombin times, secs.	30	15	166	60	14

* Read *et al.* unpublished data.

TABLE 23. *Vitamin K-Deficient Diet for Chicks*

	Gm
Soy protein	35.3
Cerelose	52.11
Vitaminized cerelose*	5.00
Salts (59A)†	5.34
Corn oil (Mazola)	1.00
DL-methionine	0.75
Glycine	0.30
Choline chloride	0.20
Total	100.00

* Vitamins provided as follows (mg/kg diet): thiamine hydrochloride 100, riboflavin 16, calcium pantothenate 20, niacin 100, pyridoxine hydrochloride 6, folic 100, inositol 100, paraaminobenzoic acid 2, B_{12} .02, biotin 0.6, ascorbic acid 250, vitamin A-acetate 10,000 IU, 20 mg of alpha-tocopheryl acetate, vitamin D_3 600 IU. † Contains in per cent: $CaCO_3$, 2.166; KH_2PO_4, 1.05 $CaHPO_4 \cdot 2H_2O$, 0.94; NaCl, 0.8; $MgSO_4$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.02; $ZnCO_3$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001.

TABLE 24. *Effect of Menadione and Beef Extract Supplements to Soy Protein Diet on Prothrombin Time of Chicks**

Supplement	Prothrombin Time, Sec.
K_3/gm diet	
0 γ	220
.01	178±11
.02	166±4
.04	113±7
.10	43±4
$Beef_3$-extract†	177±12
$Beef_6$-extract†	166±11
$Beef_0$-extract†	112±7

* Av. of 6 chicks/group. † 200 gm of beef were freeze-dried and extracted for 24 hours with alcohol and for 24 hours with ether. The solvents were evaporated off under vacuum and the fat extract was incorporated in 1 kg of the chick assay diet.

TABLE 25. *Vitamin K Deficiency in the Female Rat*

Diet	No. of Rats	Days on Experiment	Coprophagy	Number of Hemorrhagic Deaths	Prothrombin Time, Sec.
NBC casein	7	70	allowed	0	13(11–16)
NBC casein	7	90	prevented	2*	
$Beef_3$	10	50	allowed	0	13(12–16)
$Beef_3$	10	50	prevented	0	17(14–19)

* 1st death on 62nd day.

TABLE 26. *Effect of Feeding Irradiated Beef to Growing Male Compared to Female* Rats; 56 Day Experiment*

Group	No. of Rats	Sex	Method of Feeding	Coprophagy Allowed or Prevented	Deaths, %	Plasma Prothrombin Times on Surviving Rats, Sec.
1	10	male	feed intake equated with group 2	allowed	80	36.0 (28–44)
2	10	female	ad libitum	allowed	none	13.4 (12–16)
3	10	male	fed for equal gains with group 2	allowed	50	18.0 (16–21)
4	10	male	ad libitum	allowed	100	
5	15	female	ad libitum	prevented	none	17.4 (14–19)

irradiated and irradiated beef and found that these bile acids increased the absorption of vitamin K even in the case of the irradiated beef diet. However, the difference between the irradiated and nonirradiated beef was still clearly evident, as can be seen from the data in table 22.

A chick vitamin K assay was used to study quantitatively the destruction of vitamin K during irradiaton (P. B. Rama Rao, unpublished data). Since assay values could not be obtained on the fresh beef, extracts of the nonirradiated and irradiated beef were prepared. Two hundred grams of beef were freeze-dried and extracted for 24 hours with alcohol, followed by 24 hours with ether. The solvents were evaporated off under vacuum, and the fat extract was incorporated in the chick assay diet given in table 23. The results of the

assay given in table 24 show that half to three-fourths of the vitamin K present in the nonirradiated beef was destroyed by irradiation (11). Thus the chick assay is at least in qualitative agreement with the rat assay in indicating much less vitamin K in the beef following irradiation.

In order to further examine the difference in response between the male and female rat to vitamin K deficiency more extended experiments were carried out with female rats. From the data in table 25 it can be seen that even with coprophagy prevented 62 days were required for the first death of a female rat on the NBC casein synthetic ration. In another experiment a restricted feeding technique was used so that male rats (*group 1*) consumed the same amounts of food as were eaten by the ad libitum fed female rats (*group 2*), and male rates (*group 3*) made gains equal to those of female rats (*group 2*). Female rats (*group 5*) were fed ad libitum in tubular cages to prevent coprophagy. While male rats (*group 4*) fed ad libitum died of hemorrhage, none of the female rats showed symptoms of vitamin K deficiency over a 56-day period. It was observed that resistance of the female rat to vitamin K deficiency was not due to a lower food intake or to a lower growth rate (V. C. Metta, unpublished data). Dr. Mellette (unpublished data) has shown that the difference in ease of production of vitamin K deficiency is controlled by the sex hormones.

SUMMARY

From the experimental data presented it can be seen that the rat does have a dietary requirement for vitamin K which on some diets is partially supplied by coprophagy. This requirement is much greater for the male than for the female, and for the male it approximates 0.1 microgram of vitamin K per gm of ration.

Pork and beef are poor sources of vitamin K, and irradiation further lowers the available vitamin K level, thus resulting in severe vitamin K deficiency when fed as the sole source of vitamin K in the ration of the male rat (8).

This situation is entirely analogous to that of many other vitamins which are destroyed or decreased in level by processing methods, and it can be readily counteracted by providing a mixed diet containing other sources of the vitamin in question, or by supplementing the diet.

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Influence of age, sex, strain of rat and fat soluble vitamins on hemorrhagic syndromes in rats fed irradiated beef[1]

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Several investigators have now confirmed the observation of Metta and Johnson (8) that a hemorrhagic diathesis may readily develop in male rats fed certain diets containing ground beef which has received a sterilizing dose of irradiation. These observations have been extended to include other dietary regimens and current information may be interpreted as indicating that semipurified diets containing cooked irradiated beef provide a means whereby vitamin K deficiency may be readily induced in the rat, without resort to the time honored means of the administration of sulfonamides or coprophagy prevention techniques. If such an interpretation is accepted, the results to be presented here may be considered as evidence that the vitamin K requirement of the rat is influenced by the strain, sex, and age of the animal and by the level of administration of other fat-soluble vitamins.

EXPERIMENTAL CONDITIONS

The diet employed by the various investigators, whose work is included in this report, is similar to that reported by Metta *et al.* containing 35% cooked ground beef, 35% flour, 19% sucrose, 1.5% cod-liver oil, 0.5% wheat-germ oil, 4% salt mix 446, and 5% dextrose-vitamin mix. The vitamin mix is of the following composition: thiamin 2.5, riboflavin 2.5, niacin 10, pyridoxine 2.5, calcium pantothenate 20, PABA 50, inositol 100 and choline 1,000, all mg/kg dry diet. Starch has been substituted for flour in the experiments of most investigators; and salts W rather than salts 446 were used in our laboratory. Other alterations have included omission of the wheat-germ oil and cod-liver oil and substitution of more refined sources of vitamins A, D, and E.

The irradiation level for all treated beef in these experiments was 5.58 megarad. Gamma irradiation was used in all except one experiment which is so indicated.

Studies of coagulation factors have included the measurement of prothrombin time by one-stage methods of the type originally devised by Quick, and also the determination of prothrombin concentration by the method of Hjort, Rapaport and Owren (4). It is recognized that the results of the latter method may be affected by deficiencies of factor X (Stuart factor) as well as 'true prothrombin'; but, unlike the Quick test, values obtained will not be influenced by substances (i.e., factor V or fibrinogen) which are not dependent upon vitamin K. Factor V (accelerator globulin) was determined separately on all samples in our laboratory. Standard curves for coagulation factors were derived from a number of samples obtained from normal fully mature male and female rats.

Generally speaking, investigators have been consistent in the definition of hemorrhagic death. An animal was not considered to have died from hemorrhage unless there was evidence of significant hemorrhage in some area of the body on post-mortem examination, or, at least, evidence of bleeding from the heart following a cardiac puncture which yielded a sample with a markedly abnormal prothrombin level. Deaths immediately after cardiac puncture occurred rarely in the absence of these criteria.

STRAIN OF RAT

One of the first indications that various strains of rat might react differently to the irradiated beef diets was the difficulty experienced by some investigators in producing a hemorrhagic diathesis. Doisy and Matschiner, employing a strain of rats developed at St. Louis University, and Koehn (personal communication) using a strain developed at the Ft. Knox Medical Research Laboratory, were unable to induce spontaneous hemorrhagic death. Similar difficulties in his early experiments with a Texas A and M strain compared with Holtzman rats led Richardson to the conclusion that such discrepancies might be explained by strain differences.

Some of the results obtained in laboratories in which more than one strain of rat was used are shown in table 1. The data include some deaths occurring from bleeding

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TABLE 1. *Effect of Strain of Rat on Development of Hemorrhage During Irradiated Beef Diet* in Male Rats*

Investigator	Strain	Duration of Exper. Weeks	Hemorrhagic Deaths, No. Deaths/No. Observed	%
Richardson (11)	Holtzman	4	8/79	10
	Texas A & M	4	1/23	4
Doisy (3)	Sprague-Dawley	9	7/10	70
	St. Louis Univ.	13	3/46	7
Johnson (9)	Sprague-Dawley	6.6	14/20	70
	St. Louis Univ.	12.4	1/6	17
Reber (6)	Sprague-Dawley	8	18/32	56
	Holtzman	8	7/30	23

* Richardson data includes some animals receiving irradiated soybean protein. Beef irradiated at 6×10^6.

following cardiac puncture; but all may be considered to meet the criteria as outlined for the occurrence of hemorrhagic death. The St. Louis and Holtzman strains proved relatively resistant to the development of hemorrhage under the conditions of two separate laboratories. In contrast, Sprague-Dawley animals were uniformly susceptible to the beef-carbohydrate diets in all experiments shown in table 1 as well as in repeated experiments at the U. S. Army Medical Research and Nutrition Laboratory (Denver, Col.). On the other hand, the importance of the exact composition of the diet in overcoming strain susceptibility is demonstrated by the work of Blood (2) who fed diets containing 35% irradiated beef with the remainder Purina laboratory chow to Sprague Dawley rats for 2 years without evidence of hemorrhagic phenomena.

Another strain with a high degree of susceptibility to the development of hypoprothrombinemia and hemorrhagic death under our conditions is the CD strain of the Charles River Breeding Laboratories. All data in this report not otherwise qualified were obtained in our laboratory using this strain. The susceptibility of the Charles River CD rat in the present experiments is in contrast to the resistance of this strain compared with Holtzman rats to the development of vitamin K deficiency by coprophagy prevention in the experiments of Barnes and Fiala (1).

HORMONAL INFLUENCES ON THE DIETARY HEMORRHAGIC SYNDROME

The initial experiments of Metta and Johnson indicated that female rats rarely developed hemorrhagic phenomena as a result of the consumption of diets containing irradiated beef. In our investigation of this pronounced sex difference, beta irradiated and control (nonirradiated) beef diets were fed to intact and castrate male and female rats. Serial studies of prothrombin concentration by the Hjort method were performed, beginning at 14 days and continuing for as long as 20 weeks in some groups. Figure 1 illustrates the results of these studies.

Several observations may be made in regard to the animals receiving the nonirradiated beef diets. Prothrombin concentration was markedly low in initial determinations in samples from all intact male rats, with an average value of 20% of normal (range 13–23%) for samples obtained from the 14th to 34th day of feeding. In contrast, the average for samples obtained from intact females during this period was 98%, the lowest value being 77% of normal. Castrate animals had intermediate values between these two extremes. Improvement occurred with time in all groups fed the nonirradiated diets, although the intact males never reached levels which could be considered entirely normal.

A similar trend toward spontaneous improvement is notable in the groups receiving the diets containing irradiated beef with the exception of the intact males. Only the intact females were able, however, to achieve and maintain normal levels of prothrombin.

Mortality data during the first 12 weeks of this experiment are given in table 2. Hemorrhagic death had occurred in all intact males fed irradiated diets by day 34 and in all rats in both groups of castrate animals by day 63. The shorter average survival time of the castrate females compared to castrate males is consistent with the differences in prothrombin concentration found in these groups.

Occasional hemorrhage occurred in intact females fed the irradiated diets and in intact males fed nonirradiated beef diets. It is evident that irradiation of the beef is not a prerequisite for the development of low levels of prothrombin or a hemorrhagic diathesis.

The effects of castration on the hemorrhagic syndrome have been confirmed by Reber (6) and by Doisy and Matschiner (3). The reduction in mortality of male animals achieved by castration in the experiments of Reber and Malholtra is indicated in table 3, along with the effects of the administration of sex hormones by pellet implantation. Stilbestrol pellets weighing 13.6–14.6 mg and testosterone pellets weighing 10.3–16.2 mg were used. Table 3 also demonstrates that the difference between the Sprague Dawley and Holtzman strains is consistent through the various treatments given.

We have investigated the effects of estrogen administration in the adult male rat (av. wt. 415 gm) fed an ir-

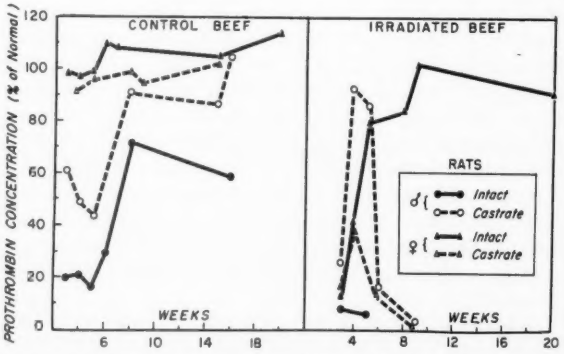

FIG. 1. Prothrombin concentration (method of Hjort *et al.*) during feeding of nonirradiated and irradiated (5.58 megarad) beef diets to intact and castrate male and female rats.

TABLE 2. *Hemorrhagic Deaths During 12 Weeks of Beef Diets 10 Rats per Group*

	Weeks of Feeding				Total Hemorrhagic Deaths	Ave. Survival Time of Animals Dying of Hemorrhage, Days
	1–3	4–6	7–9	10–12		
Nonirradiated beef diets						
Intact (M)	0	1	1	0	2	45
Castrate (M)	0	0	0	0	0	
Intact (F)	0	0	0	0	0	
Castrate (F)	0	0	0	0	0	
Irradiated beef diets						
Intact (M)	8	2			10	17
Castrate (M)	4	2	4		10	40
Intact (F)	2	0	1	0	3	33
Castrate (F)	5	3	2		10	29

TABLE 3. *Effect of Sex Hormones on Mortality of Male Rats During Irradiated Beef Diets Begun at Weaning*

Treatment	% Mortality—5 Week Period	
	Sprague-Dawley	Holtzman
Intact male	62	7
Castrate	37	0
Intact + testosterone	88	27
Castrate + testosterone	70	28
Intact + stilbestrol	0	0
Castrate + stilbestrol	0	0

Data of Reber *et al.*, 1959–60.

radiated beef diet. Death occurred in 2 of the 5 animals which received 50 micrograms of estradiol benzoate in sesame oil at 3-day intervals over an 18-day period, compared to 100% mortality within 13 days in the control group receiving sesame oil only. Prothrombin concentration on day 3 averaged 28% in the estrogen-treated group compared to less than 12% of normal in the animals receiving the sesame oil diluent. Factor V levels decreased somewhat from preliminary values in both groups and no change occurred in the average fibrinogen levels of either group. Since both factor V and fibrinogen have been reported to be affected by estrogen administration, it is interesting to note that neither substance can be implicated as responsible for differences between animals in the experiment described. It should be recognized, however, that experiments in which estrogens are administered to animals on ad libitum diets are difficult to evaluate because of the anorexogenic effect of these substances and the resultant decrease in food consumption and weight gain in treated animals.

In a further experiment we undertook to decrease the resistance of the adult female rat to the development of hemorrhage by the injection of androgens. Animals selected had been receiving one of three types of diets: nonirradiated beef diets, irradiated beef diets, or Purina Dog Checkers, for at least a month prior to the beginning of hormone injections. Each diet group was subdivided equally into animals receiving injections of sesame oil only and animals which were given 1 mg of testosterone propionate in 0.2 cc sesame oil twice weekly. Preliminary prothrombin levels averaged 117 and 111% of normal for the groups receiving the nonirradiated beef diets and the stock laboratory diet, respectively. Animals which had been fed for a month on irradiated beef diets began the new experiment at a somewhat lower level: 95% of normal prothrombin. During a 6-week experimental period, in which coagulation factors were determined weekly, no significant change occurred in average values for prothrombin in either sesame oil or testosterone treated animals receiving nonirradiated beef or stock laboratory diets, although an occasional moderately low level was observed in rats receiving testosterone. In contrast, further decline in prothrombin levels occurred in all animals on irradiated beef diets. At 2 weeks, average values were 75% for sesame oil-controls and 57% for rats receiving testosterone. Mortality data revealed a

TABLE 4. *Effect of Age at Onset of Irradiated Beef Feeding on Prothrombin Levels and Hemorrhagic Mortality in Male and Female Rats*

Age, Weeks	No. Rats Observed	Av. Prothrombin, % Normal	Hemorrhagic Mortality 4 Weeks, %
Males:			
3–5	57	32	41
12–15	31	26	77
15–27	19	15	100
Females:			
3–5	32	64	12
10–12	10	104	0
30	5	110	0

TABLE 5 *Effect of Tocopherol on Development of the Hemorrhagic Syndrome in Rats Fed Beef Diets*

Alpha Tocopherol mg 2×/wk	No. Rats	Prothrombin, %	% Rats Hemorrhagic Day 0–14
Irrad. diets			
0	37	43	22
5	37	49	38
50	37	48	46
200	17	13	75 Day 0–23
Control diets			
0	6	112	17
5	11	97	18
50	11	80	18
200	11	46	55

TABLE 6. *Effect of Bile Acids or Tween-80 in Male Rats Fed Beef Diets*

Diet	% Mortality, 10 Weeks Bile Acids		Prothrombin Time, Seconds Bile Acids	
	0	125*	0	125*
Non-Irrad. beef	20	0	30	15
Irradiated beef	100	60	166	36
Irrad. beef + 0.25%	70		80	

* Equal weights of cholic acid, desoxycholic and lithocholic acids for total of 125 mg consumed per day. Data of Read, Witt, *et al.*, 1959–60.

somewhat greater difference: 2 of the original 7 (29%) of the sesame oil group and 5 (71%) of those receiving testosterone had died at the 4-week point of the experiment. These observations in female rats are consistent with those of Reber's studies in male animals in indicating a deleterious effect of the administration of androgens in animals receiving irradiated beef diets. Average values for factor V were lower in animals receiving testosterone in all three diet groups. Deficiency of this factor may have contributed to the deaths of animals in which prothrombin was concomitantly low, but abnormal levels of factor V were not shown to be associated with an increased mortality in animals with normal values for prothrombin.

In keeping with the demonstrated importance of hormonal factors in the development of hypoprothrombinemia and hemorrhagic death, it would be expected that predictable differences in response to the diets being studied should occur at appropriate stages of maturity in male and female animals. The data shown in table 4, compiled from various experiments under comparable conditions in our laboratory, indicate an increasing susceptibility to hypoprothrombinemia and hemorrhage with maturity in male animals and the reverse situation in female animals.

Recent observations indicate that such differences in prothrombin concentration between the sexes are not limited to animals fed irradiated beef diets but may also be demonstrated in Charles River CD strain rats fed stock laboratory chow without additional vitamin K supplementation. Approximately half of 175 samples obtained from male animals from weaning to 150 days of age revealed low levels of prothrombin whereas female animals, with low levels occurring not infrequently during the first 2 months, demonstrated consistent improvement with maturity. The rate of growth was not directly related to the prothrombin level in that low levels persisted in male animals even after weight gains had stabilized at a rate equal to or less that of female rats.

FAT SOLUBLE VITAMINS

Removal of cod-liver oil from the diets being studied was found by the Illinois investigators to result in a moderate decrease in the incidence of hemorrhagic deaths. Doisy and Matschiner had further observed that substitution of their usual plan of micronutrient supplementation for that used by Johnson's group resulted in better maintenance of prothrombin. One major difference in these two regimens was the level of supplementation of vitamin A. Exploring this lead further, these investigators confirmed the previously reported hypoprothrombinemic effect of large doses of vitamin A (5) and found that animals receiving diets containing irradiated beef were more susceptible to the effects of increasing amounts of vitamin A than rats fed purified diets containing casein as the source of protein (7).

Early studies in our laboratory to determine the possible role of vitamin E in the dietary hemorrhagic syndrome indicated that vitamin E deficiency, as indicated by dialuric acid hemolysis of erythrocytes, occurred frequently in animals fed for 12–20 weeks on diets containing irradiated beef and 0.5% wheat germ oil. Similar evidence for the destruction of tocopherol activity in diets containing irradiated foods has accumulated from several sources, one of the earliest reports being that of Poling *et al.* (10).

In short term experiments, however, increases in the level of tocopherol, administered as a separate oral supplement, resulted in a deleterious effect similar to that just mentioned for increasing amounts of vitamin A. As indicated in table 5, an increase in mortality and declining levels of prothrombin occurred at the higher doses of tocopherol in animals fed nonirradiated as well as irradiated beef diets.

There is evidence to suggest that inefficient absorption of vitamins, i.e. vitamin K, from the intestinal tract may contribute to the development of a deficiency state. The observations of Read *et al.* (12) indicate that improvement in survival and in prothrombin times of rats fed irradiated beef diets occurred when bile acids were added to the diets. Some of the results of these experiments are illustrated in table 6, which includes data indicating that some beneficial effect may also follow the administration of the emulsifying agent Tween-80.

In titration studies of vitamin K requirement, conducted in the Denver laboratory, it was further found that the menadione requirement for the maintenance of normal prothrombin time was decreased from 4 to 2 micrograms per day by the administration of 83 mg of mixed bile acids.

SUMMARY

It has been shown by a number of investigators working on this problem that the incidence of a hemorrhagic syndrome associated with the feeding of irradiated beef diets to the rat is influenced by the age, sex, and strain of rat employed. Administration of male hormones or castration of female rats results in an increased susceptibility to hypoprothrombinemia and hemorrhage; whereas the converse is true for the administration of estrogenic hormones or castration of the male animal. Changes in the composition of the diet, particularly the addition of large amounts of vitamins A and E, may increase mortality and depress prothrombin levels. Supplemental bile acids or an emulsifying agent have a beneficial effect, probably secondary to improved absorption of vitamin K.

Irradiation of the beef in the diets which have been studied is not a prerequisite for the development of low levels of prothrombin in the male rat. Hypoprothrombinemia may occur in animals fed similar diets containing nonirradiated beef and even in animals receiving a commercial stock laboratory chow.

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Tissue modification and irradiated food ingestion[1]

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Long-term feeding studies with rats designed to evaluate the wholesomeness of irradiated organ meats, pork, jam, carrots, peaches, and flour have been completed or are in progress in this laboratory. The details concerning the diet composition, irradiation levels and sources, and animal selection have been published (3, 4). With organ meats and pork no statistical treatment differences were shown in growth, breeding and longevity. Similar results, recently completed, have been also shown with jam, carrots, and peaches (unpublished observations).

In addition to the visible physiological function data, an extensive gross and histopathological examination of the tissues of the dead or sacrificed animals has been carried out. A detailed procedural guide established by the Armed Forces Institute of Pathology for the irradiated foods program was followed for the examinations. Twenty-two tissues, with several levels of some, were studied.

A summation of the pathological findings on the animals comprising the first generation ingesting the pork diet are presented here. This group was made up of 156 animals, 78 males (26 from each food group; control, 2.79 and 5.58 megarad), and a similar group of females. Seventy-six animals or 48.7% survived the 730-day longevity period and were sacrificed. Twelve animals were found dead in their cages and histopathological examination was not possible. Gross examination was made on eight of these, the remaining four were so extensively decomposed that even gross was not possible. The remainder of the group were sacrificed as outward signs indicated imminent death. These signs were, extensive loss of appetite and weight, heavy breathing, inner ear instability and visible abnormal growths.

Table 1 is a summation of the causes of death or pathological conditions found upon sacrifice. Chronic progressive nodular pneumonia is the most common cause of death or most apparent condition on sacrifice. No treatment patterns are noticed. A total of 24 benign or malignant tumors were found. Eleven of these were of such size or nature as to cause death or change the course of the animals metabolism. All the tumors found are listed on table 2.

A second area of investigation at the tissue level consists of those studies designed to determine if the ingestion of rations containing irradiated foods produces changes in enzyme activity. These studies have been based on the premise that any gross physiological or pathological change is a manifestation of disturbed metabolism at the cellular level. Hence, it is probable that detectable enzymatic changes might be observed before more extensive physiological and pathological changes become apparent.

Animals raised on jam rations consumed considerable

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TABLE 1. *Causes of Death or Pathological Conditions Found on Sacrifice—Irradiated or Control Pork Diet—First Generation Animals*

Conditions Observed	Pork Irradiation Level—Megarad					
	Female			Male		
	0	2.79	5.58	0	2.79	5.58
No significant lesions	11	6	6	10	15	11
Chronic progressive nodular pneumonia	9	12	9	10	7	11
Middle ear infection with brain involvement	1	0	2	1	1	3
Pituitary hemorrhage	1	1	2	1	0	0
Dental anomalies	1	0	2	0	0	0
Septicemia	0	2	1	0	0	0
Unknown—examination not possible	1	1	1	0	1	0
Other causes	1	3	1	0	0	0
Tumors	1	1	2	4	2	1
Total number of animals	26	26	26	26	26	26

TABLE 2. *Tumor Incidence**

Mammary adenoma	1 F (0)
Mammary fibroadenoma	2 F (0), 2 F (2.79)
Pituitary adenoma	3 M (0), 1 F (0), 1 M (2.79), 1 F (2.79), 2 F (5.58)
Pituitary carcinoma	1 M (2.79), 1 F (2.79), 1 M (5.58)
Uterine fibroadenoma	1 F (2.79)
Adrenal—unknown	1 M (0)
Adrenal—adenofibroma	2 M (5.58)
Spleen lymphosarcoma	1 M (0)
Lipoma peritoneum	1 F (2.79)
Fibroma (front leg)	1 F (2.79)
Testicle-sertoli cell tumor	1 M (5.58)

* The figure in parentheses refers to the irradiation level of the ration on which the animal was raised.

quantities of irradiated sucrose. Consequently, studies were made of the rate of oxidation of various hexoses and hexose phosphates in heart and kidney homogenates prepared from these animals. The reaction medium contained exogenous diphosphopyridinenucleotide and such systems have been shown to be capable of oxidizing glucose and fructose to carbon dioxide (16). Heart homogenates from animals raised on rations containing irradiated jam showed a decrease in the rate of oxidation of fructose (table 3). This effect was observed in the second and third generations but not the fourth generation animals. An explanation for this situation might be that the concentration of fructose in the irradiated jam ration is considerably reduced by the irradiation and subsequent storage of the jam and results in a corresponding decrease in the rate at which the tissue is able to oxidize fructose.

It was initially reported by Kraybill, Read and Friedemann (6) that liver homogenates prepared from male rats, raised on an irradiated composite ration, showed a significantly higher cytochrome oxidase activity than those homogenates prepared from animals raised on the unirradiated control ration. The composite ration contained considerable quantities of beef, ham and bacon. This ration was fed to four generations and in all cases this difference was observed in male but not female animals (12). Subsequent studies demonstrated that a comparable modification of cytochrome oxidase activity was produced by feeding rats a ration containing irradiated beef at a level of 35% on a dry weight basis (13). In both the beef ration and composite ration the irradiated meat was not cooked prior to incorporation into the ration. The additional variables of storage at elevated temperature and cooking of irradiated beef before incorporation into the ration were also evaluated. A significant increase in liver cytochrome oxidase activity was produced by feeding rations containing beef which had been irradiated to 5.58 megarad and stored for 3 to 6 months at 70°F. A similar difference associated with the feeding of a ration irradiated to a level of 2.79 megarad was observed only after storage for 3 to 6 months and cooking. Rats fed rations containing irradiated uncooked pork did not show an increase in cytochrome oxidase activity.

Experiments in our laboratories have demonstrated that the feeding of a ration contained cooked, stored, irradiated pork (5.58 megarad) produces an increase in cytochrome oxidase activity. This increase was observed in liver, heart and kidney homogenates as well as in liver mitochondrial preparations (table 4). These assays were carried out on male animals which had been fed the irradiated ration for 10 to 12 weeks. Similar results were observed using female animals. However, no change in liver cytochrome oxidase activity was observed in male animals which had been raised on the irradiated pork ration for 8 to 9 months.

TABLE 3. *Fructose Oxidation in Heart Homogenates From Rats Raised on Rations Containing Control and Irradiated (5.58 megarad) Jam*

	μl Oxygen/hr/mgN*	
	Second generation	Third generation
Control	330±3	193±14
Irradiated	186±2	156±24

* Mean and standard deviation.

TABLE 4. *Cytochrome Oxidase in Tissues of Rats Raised on Rations Containing Control and Irradiated (5.58 megarad) Pork*

	μMole Oxygen/mgN/hr*	
	Control	Irradiated
Kidney homogenate	203±18	240±20
Heart homogenate	333±15	400±56
Liver homogenate	197±20	230±19
Liver—mitochondria	364±31	429±36

* Mean and standard deviation.

TABLE 5. *Adenosinetriphosphatase Activity of Liver Mitochondria From Rats Raised on Control and Irradiated (5.58 megarad) Pork*

	μMole Inorganic Phosphate Released/20 min/mgN*	
	Endogenous	Dinitrophenol-Stimulated
Control	2.0±0.8	16.1±3.7
Irradiated	1.6±0.7	22.9±2.8

* Mean and standard deviation.

Read and co-workers (17) have also studied the effect on liver cytochrome oxidase of feeding rations containing irradiated (5.58 megarad) beef udder fat which has been stored for 3 months at 75°F. before incorporation into the ration. Feeding a ration containing 30 % irradiated fat resulted in an increase in liver cytochrome oxidase, after the animals had been fed the ration for a period of 3 to 4 months. No change in liver cytochrome oxidase resulted from feeding a ration containing the irradiated fat at a level of 10%. It is also of interest that if the corn oil is omitted from the beef ration, the cytochrome oxidase activity of liver homogenates from animals on the unirradiated ration increased to a level equal to that observed in animals raised on the rations containing the irradiated beef (17).

It has been shown by Metta, Mameesh and Johnson (10) that a ration containing irradiated cooked beef but no exogenous vitamin K produced hemorrhagic lesions, particularly in male animals. Oral supplements of vitamin K alleviated this condition. Read (personal communication) has recently studied the liver cytochrome oxidase and prothrombin level in animals raised on Johnson type irradiated beef rations, with and without oral supplements of vitamin K. Unsupplemented rations resulted in the elevation of both cytochrome oxidase activity and prothrombin time. An oral supplement of 0.5 γ of vitamin K per day was effective in restoring cytochrome oxidase activity to the same level as that observed in animals fed the control rations. The oral supplement of vitamin K also reduced the prothrombin time to normal levels. These experiments would thus indicate some relation between cytochrome oxidase activity and vitamin K nutrition.

The metabolic role of vitamin K has not yet been established; however, there is some evidence that would implicate vitamin K in the oxidative phosphorylation process. Mitochondrial preparations from vitamin K deficient chicks showed decreased P/O ratios (9) and increased DPNH-oxidase (5). Mitochondrial preparations from livers of rats fed irradiated pork rations showed no change in P/O ratios or DPNH-oxidase activity compared to similar preparations from rats fed the control ration. However, liver mitochondrial preparations from the irradiated pork fed animals showed an increase in dinitrophenol-stimulated adenosinetriphosphatase (table 5).

DISCUSSION

It is of interest to note some factors which are common to the incidence of the hemorrhagic syndrome as well as to the elevation of cytochrome oxidase activity: *a*) both conditions were observed in rats raised on rations containing irradiated meats; *b*) in both cases males are more susceptible than females; and *c*) both conditions were alleviated by vitamin K supplements. In addition, it must be noted that an increase in cytochrome oxidase activity was initially observed in animals which showed no physiological lesions or change in over-all performance and which received exogenous vitamin K in their rations. On this basis one could conclude that the modification of cytochrome oxidase activity is more sensitive to vitamin K stress than the incidence of the hemorrhagic disorders.

The specific metabolic role of vitamin K has not been elucidated. Numerous reports have associated vitamin K with the oxidative phosphorylation system (1, 2). More specifically there has been the suggestion that vitamin K acts between diphosphopyridinenucleotide and cytochrome b in the electron transport chain (8), however this hypothesis has not been confirmed as yet. These experiments would be the first to indicate some relationship between cytochrome oxidase and vitamin K. To explain an increase in cytochrome oxidase activity associated with a deficiency of vitamin K one would have to postulate that a deficiency in vitamin K resulted in an uncoupling effect producing an increase in the rate of the oxidative reaction. The observed increase in dinitrophenol-stimulated adenosinetriphosphatase would be consistent with this theory. Vitamin K would thus be implicated in the high energy phosphate transfer rather than the electron transport system.

If the increase in cytochrome oxidase activity observed in animals raised on rations containing added vitamin K is indeed due to a vitamin K deficiency one must conclude that the dietary vitamin K is unavailable to the animal. This could be the result of a binding or degradation of the vitamin in the prepared ration. Oxidative degradation of vitamin K could be quite appreciable in a ration containing considerable amounts of oxidized lipides as is the case in the irradiated beef and pork rations. Another possibility is that the intestinal absorption of vitamin K is retarded in some fashion. Schreiber and Nasset (14) have demonstrated that irradiation considerably reduces the rate of absorption of lard. In such a situation one could also expect a reduction in the rate of absorption of fat soluble vitamins. Such a situation could exist in the irradiated beef and pork rations which contain considerable amounts of irradiated lipides. It has also been proposed (11) that the vitamin K deficiency is due to the fact that animals fed the irradiated rations do not practice coprophagy and, hence, the vitamin K produced from intestinal synthesis is not available.

Several other factors should be considered in regard

to the cytochrome oxidase phenomenon. It has been shown that a diet deficient in essential fatty acids produces an increase in cytochrome oxidase activity (15). It does not appear that essential fatty acids are the limiting factor in these experiments since elevated cytochrome oxidase activity has been observed in animals raised on beef rations which contained sufficient corn oil to provide the essential fatty acids. Also no change in cytochrome oxidase was observed in a ration containing 10% irradiated beef udder fat as the only lipide source. This ration should have accentuated any symptoms which could be attributed to essential fatty acid deficiency. It is also significant that the composition of a purified preparation of cytochrome oxidase was shown to be 33% lipide (7). If a change in the nature of the dietary lipides resulted in a corresponding change in the lipide of the cytochrome oxidase complex it would not be unreasonable to expect some change in the enzyme activity as a consequence.

A more satisfactory explanation of this phenomenon must await a better understanding of the mechanism of the cytochrome oxidase system as it is related to vitamin K metabolism and lipide composition.

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Human feeding studies with irradiated foods[1]

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During the past 7 years the U.S. Army Quartermaster Corps has undertaken a comprehensive study of the possible uses of ionizing radiation in the processing of food (9). As a part of this program the Army Medical Service has supervised the testing of the wholesomeness of food treated with ionizing radiation. Wholesomeness testing outlined by the Food and Drug Administration (4) includes two types of studies; first, the evaluation of the nutritional adequacy, and second, of the potential toxicity of such irradiated foods. As a final test of toxicity, a program of human feeding tests was undertaken at this laboratory.

[1] The opinions expressed are those of the author and are not necessarily endorsed by the Department of Defense.

METHODS

Human feeding tests were divided into three phases. First, after a series of foods had successfully passed subacute toxicity tests in animals, the foods, stored frozen after irradiation, were fed for periods of 15 days to humans. Second, a more restricted group of the same foods undergoing chronic toxicity tests in animals were fed to humans after irradiation and a period of at least 3-months' storage at room temperature. A third phase, long-term human feeding, will be performed when the chronic toxicity tests in animals have been completed.

The foods tested in humans are listed in table 1. Those tested in the second phase were selected to give examples of different types of food for which radiation processing

TABLE 1. *Irradiated Foods Tested in Humans**

Meat	
Bacon† (1, 2)	Chicken stew† (1)
Corned beef (2)	Frankfurters (2)
Ground beef (2)	Ground ham (2)
Beef steak (2)	Ham stead (2)
Chicken† (2)	Ground pork† (2)
	Sausage (2)
Fish	
Cod† (1)	Salmon (2)
Haddock (2)	Shrimp† (1, 2)
	Tuna† (1, 2)
Vegetables	
Asparagus (2)	Cauliflower (2)
Green beans† (2)	Celery (2)
Lima beams (2)	Cole slaw† (2)
Beets (2)	Corn† (2)
Brussel sprouts (2)	Mushrooms (2)
Cabbage (2)	Peas (2)
Carrots† (2)	Sweet potatoes† (2, 6)
	White potatoes† (6, 7)
Fruits	
Dried apricots (2)	Oranges† (4)
Cherries (2)	Orange juice† (3)
Dried fruit compote† (1)	Peaches† (2)
Melon balls (2)	Dried pears (2)
	Strawberries (2)
Cereal products	
Bread (2)	Macaroni (2)
Crackers (2)	Nut roll (2)
Cereal bar (2)	Pound cake (2)
Flour† (5)	Rice (2)
Miscellaneous	
Dessert powder (2)	Pineapple jam† (2)
Powdered whole milk (2)	Strawberry jam (2)
Peanut butter (2)	Sugar (2)

* Numbers after each food refer to the radiation dosage: (1) 4,000,000 r. (2) 3,000,000 r. (3) 2,500,000 r. (4) 150,000 r. (5) 80,000 r. (6) 20,000 r. (7) 10,000 r. † Foods tested after room temperature storage.

appeared to offer advantages for sterilization, 'pasteurization,' insect deinfestation, and sprout inhibition. These foods were placed in sealed cans, frozen, and irradiated by gamma rays from spent reactor fuel rods. The radiation dosage varied from 3 to 4 million rep for sterilization, to about 100,000 rep for 'pasteurization,' to 10,000 rep for sprout inhibition. In all studies except one, control foods were obtained from the same procurement lot, sealed in cans, and held frozen until use.

Seven separate feeding tests were carried out, each with nine or ten test subjects. The number of irradiated foods used and the level of dietary calories supplied by them varied, (table 2). Only one irradiated food was employed, ground pork, in the fifth test. In this case the control food was not of the same procurement lot, but was obtained locally. For each study, the subjects ate irradiated foods for a period of 15 days. In six of the tests, half of the subjects received the diet of irradiated foods for 15 days followed by the diet of control food for another 15 days; the rest of the men consumed the diets in

TABLE 2. *Human Feeding Tests*

Test	% of Calories from Irradiated Foods	No. of Irradiated Foods	Storage Condition	Duration of Test, Days
1	35	11	Frozen	15
2	60	17	Frozen	15
3	80	29	Frozen	15
4	100	30	Frozen	15
5	32	1	Room Temp.	15
6	80	8	Room Temp.	15
7	80	14	Room Temp.	15

TABLE 3. *Individual Exposure to Irradiated Foods*

No. of Rations of Irradiated Foods Consumed	No. of Subjects	Period of Consumption, Months
less than 10	8	1
11–20	9	1–11
21–30	8	5–13
31–40	6	13
over 40	2	12

reverse order. In the sixth test the irradiated and control diets were fed alternately for 7-day periods.

Before the irradiated foods stored at room temperature were fed samples were tested bacteriologically for sterility, and injected into mice to test for the presence of bacterial exotoxin. All foods were sterile and contained no exotoxins.

The test subjects were a group of young men, aged 19 to 24. They were members of the Mennonite faith, conscientious objectors, who volunteered for service as research subjects to fulfill their obligations to the Selective Service System. A total of 33 men participated in these tests. The men were housed on a metabolic ward where careful supervision was maintained of food intake, activity, and collection of excreta.

During each study, and for a period of 1 year thereafter, each subject was carefully examined medically, and received a series of clinical laboratory tests of hematologic, liver and kidney function. These tests included prothrombin times during and after the last three feeding trials. In some of the studies measurements were made of the digestibility of the macronutrients and of the metabolizable energy of the diets. Also, the urinary excretion of total glucuronides, total ketones and total organic acids was measured in certain tests to determine the presence of potentially toxic compounds.

Evaluations were also made of the acceptability of the irradiated foods. Although differences were found between the control and irradiated foods, these results are omitted because the nature of the present studies did not permit a satisfactory test. The details of foods tested, menus, cookery, and laboratory procedures, together with the detailed results of these seven trials are reported elsewhere (1–3, 5, 7).

RESULTS

No toxic effects whatever from irradiated foods were observed in the human test subjects either during feeding

or afterward. There were no significant differences in urinary excretion of total glucuronides, total ketones or total organic acids between subjects receiving irradiated versus control diets. The digestibilities of the macronutrients and the metabolizable energy were similar in the irradiated and control diets.

DISCUSSION

It must be noted that individual subjects consumed varying amounts of irradiated foods, since some participated in more tests than others, while the tests themselves provided differing proportions of the caloric intake from irradiated foods. Therefore, the exposure of each individual to potentially toxic compounds in radiated foods varied. For each subject the total intake of radiated foods was converted to rations (one ration is one man's daily food intake). The total consumption of irradiated foods by individuals is described in table 3, which also lists the length of time between first and last consumption. The maximum exposure of an individual to irradiated food was only 41 rations consumed at intervals over a period of 12 months.

The fifth feeding test is of special interest for the wholesomeness program. Metta and co-workers (6) reported that a diet of 35% irradiated beef solids produced death from hemorrhage in rats. The syndrome could be prevented by the administration of vitamin K. These findings were confirmed at this laboratory (8) and extended to show that pork diets also produced the hemorrhagic syndrome even more rapidly than beef. In the fifth human feeding test reported here irradiated pork was fed to humans at this same level of the diet without supplementation with vitamin K. Not only were no clinical effects produced, but the blood prothrombin times were not prolonged even slightly from normal.

SUMMARY

A group of young men consumed diets consisting of up to 100% irradiated foods for periods of 15 days in a series of seven tests. Some 54 different irradiated foods, were either frozen or at room temperature, after irradiation, tested stored. There was no evidence of toxic effects from eating irradiated foods in any of the men, either by clinical examination or by laboratory tests.

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A summary of the wholesomeness of gamma-irradiated foods[1]

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UTILIZATION OF IONIZING RADIATIONS for the preservation of foods has been an intriguing possibility for nearly 15 years. Because of its unique promise, an extensive research program of the potential application has been in progress for nearly 10 years sponsored primarily by the Department of Defense. This program has encompassed two major areas of interest: the technological feasibility of the process, and the suitability of the products for human consumption. The latter area is commonly referred to as 'wholesomeness' and in its broadest sense forms the basis for the present symposium.

Wholesomeness also includes two subdivisions: potential toxicity and nutritional adequacy. Although these facets are difficult to separate experimentally, techniques for achieving this have been outlined by Lehman and Laug (22). The application of these techniques is amply demonstrated by the presentations during this symposium as well as in the reports from an earlier symposium presented before this society 4 years ago (16).

In a program of this magnitude, it is not surprising that difficulties have arisen. Several investigators have uncovered problem areas requiring more detailed investigations. These problems have been found in both the toxicological and nutritional adequacy experiments but, by and large, they have been resolved as nutritional in origin. Nevertheless, several remain under active investigation. Regardless of the final decisions concerning the wholesomeness of radiation-preserved foods, these investigations have contributed toward furthering our knowledge concerning nutrition; in this sense, their importance may transcend the original goal of the research project.

RESULTS OF TOXICITY TESTS

Before examining in detail the nutritional problems which have arisen, it is timely to consider briefly the results of the many individual toxicity tests. As seen in table 1, 22 foods are under investigation at the present time using four different animal species plus man. These foods were originally selected as representative of a much wider spectrum of products and also because they could constitute a nonmonotonous diet for extended human feeding studies. Since earlier studies (4, 7, 35, 37, 39, 46) had shown that the irradiation process, per se, did not produce acutely toxic compounds in foods, the present experiments employ foods treated with two dosage levels and then stored under 'normal' conditions for several months following irradiation. Before diet formulation, the foods are prepared as for table use. Thus, only cabbage, jam, oranges, and peaches are fed raw following storage.

Among the rat feeding trials, seven studies have been completed including histological examination of tissues from all animals. No significant differences between the control and irradiated diet fed animals were observed with green beans (41, 44), chicken (41, 44), corn (33), evaporated milk (13), pork loin (8), white potatoes (6), and tuna fish (33). Eight additional foods have shown no adverse effects upon growth, reproduction, life span, or disease incidence as a result of radiation treatment: bacon (30), beef stew (13), codfish (1), dried fruit mixture (30), oranges (34), sweet potatoes (1), peaches (9), and shrimp (34). In these latter cases, the accompanying histological examinations are still in progress.

Sixteen of the 22 foods have now been fed to dogs (beagles) for 2-year periods, including growth and at least two breeding trials. No deleterious effects of radiation processing have been observed under these conditions for bacon (17), green beans (21), beef (40), beef stew (13), cabbage (18), carrots (14), chicken stew (24), codfish (1), corn (25), flour (40), dried fruit mixture (21), evaporated milk (12), pork loin (26), sweet potatoes (1), shrimp (14), and tuna fish (27). For eight of these (bacon, green beans, cabbage, chicken stew, corn, dried fruit mixture, pork loin, and tuna fish) the histology phase has also been completed. It should be noted that difficulties encountered in reproduction by dogs fed irradiated beef diets are discussed in detail in a separate paper on this symposium (28). Neither of the monkey

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experiments have progressed far enough yet for evaluation of the data.

All of these 2-year chronic toxicity experiments include observations of cancer incidence. Nevertheless, more detailed investigations into potential carcinogenicity are in progress employing tumor susceptible strains of mice. In one of these (29), diets containing 10% fat from fried irradiated (5.58 megarad) or nonirradiated uncured bacon have been fed throughout the life span of AHe and C_3H strains of mice. No significant differences in the rate of growth or incidence of spontaneous mammary carcinoma or hepatoma were seen between the two experimental groups. Similarly, experiments (20) with mixtures of naturally occurring sterols or of beef, fish, milk and cheese extracts as well as vegetable oil or lard have failed to show an increased tumor incidence following extended feeding, painting or injection of the irradiated materials (10^5 to 10^7 rads). None of the additional composite diet mouse feeding experiments have progressed far enough for adequate evaluation.

With the exception of the mouse carcinogenicity experiments, the animal feeding trials incorporate only one or two individual foods. However, a little information is available concerning the consumption of totally irradiated diets. Several experiments utilizing commercial animal feeds (10, 11) or semisynthetic rations (23, 42, 43) have been reported. No toxic effects were observed although reports of radiation-induced vitamin destruction have been frequent. In an experiment (38) employing composite diets of nine raw frozen-stored irradiated (5.58 megarad) or nonirradiated foods no toxic symptoms were observed in four successive generations of rats. Similar information concerning cooked room temperature-stored irradiated diets might also be useful.

TABLE 1. *Food Items and Species for Toxicity Testing*

Food Item	Radiation Dose Megarad	Storage Temperature	Species and Status*			
			Rat	Dog	Monkey	Mouse
Bacon	2.79 and 5.58	Ambient	F	C		C
Beans, green	2.79 and 5.58	Ambient	C	F		P
Beef, ground	2.79 and 5.58	Ambient	P	F		P
Beef stew	2.79 and 5.58	Ambient	F	F		P
Cabbage	0.28 and 0.56	4°C	P	C		P
Carrots	2.79 and 5.58	Ambient	P	F		P
Chicken	2.79 and 5.58	Ambient	C	P		P
Chicken stew	2.79 and 5.58	Ambient	P	C		P
Codfish	2.79 and 5.58	Ambient	F	F		P
Corn	2.79 and 5.58	Ambient	C	C		P
Eggs, powdered	0.93 and 2.79	Ambient		F		
Flour	2.79 and 5.58	Ambient	P	F		P
Fruit, dried	2.79 and 5.58	Ambient	F	F		P
Jam, pineapple	2.79 and 5.58	Ambient	P	P		P
Milk, evaporated	2.79 and 5.58	Ambient	C	F		P
Oranges	0.18 and 0.28	4°C	F		P	
Peaches	2.79 and 5.58	Ambient	F		P	P
Pork loin	2.79 and 5.58	Ambient	C	C		P
Potatoes, sweet, canned	2.79 and 5.58	Ambient	F	F		P
Potatoes, white	0.013–0.020 and 0.027–0.040	4°C	C	P		P
Shrimp	2.79 and 5.58	Ambient	F	F		
Tunafish	2.79 and 5.58	Ambient	C	C		P

* Notations used: P: experiment in progress, data incomplete; F: feeding phases completed, pathology incomplete; C: experiment complete including pathology.

TABLE 2. *Feed Efficiency of Weanling Rats Fed Gamma-irradiated Proteins as 9% of a Purified Diet*

Radiation Dose (Megarad)	Grams Gain/Gram Consumed				
	0	2.79	5.58	9.30	18.60
Protein:					
Lactalbumin	0.28	0.28	0.27		
Egg albumin	0.28	0.28	0.29		
Casein	0.28	0.27	0.25		
Wheat glutin*	0.08			0.09	0.00
Soybean alpha-protein†	0.00	0.17	0.17	0.00	0.04
Zein‡	0.00			0.00	0.00
Shrimp (shelled, deveined, enzyme inactivated)	0.27	0.25	0.28		

* Diets supplemented with 0.60% L-lysine-HCl and 0.045% DL-tryptophan. † Diet supplemented with 0.3% cysteine. ‡ Diet supplemented with 3% casein at the expense of sucrose.

NUTRITIONAL ADEQUACY

It is generally recognized that radiation sterilization of food does not significantly alter the nutritional value of the macronutrients. However, recent data obtained by Windmueller, Ackerman, and Engel (unpublished data obtained under contract, see reference (14)) suggests that radiation may improve the utilization of at least one protein. Rat feeding experiments employing diets containing 9% of the gamma-irradiated dry protein yielded the data presented in table 2. It is interesting to note that the growth of rats raised on soybean α-protein diets was enhanced following either 2.79 or 5.58 megarad irradiation treatment. However, no beneficial effect of 9.3 or 18.6 megarad treatment was obtained. Inasmuch as analogous growth improvement was observed in rats fed heated or ethylene oxide treated soybean protein, these investigators conclude that 2.79 or 5.58 megarad irradiation must have inactivated the trypsin inhibitor found in soybeans. If so, then the higher radiation doses must have decreased the protein quality so as to offset the improvement resulting from inactivation of the trypsin inhibitor. Irradiation (2.79 megarad) has been shown not to inactivate the lima bean trypsin inhibitor (31). However, others (15) report that the trypsin inhibitor from lima beans is more resistant to denaturation than is that from soybeans.

TABLE 3. *Composite Diet Formulation, Radiation Levels, Cooking Conditions, and Comparison to Military "A" Ration*

Food Item	Radiation Levels (Megarad) for Diet Codes			Cooking Conditions	Composition as % Dry Solids	
	0	×	2×		Experimental diets	Military master menu 1956–57
Bacon, cured	0	2.79	5.58	Baked	2.6	17.8% meats, poultry, fish and specialty items
Beef, ground	0	2.79	5.58	Baked	8.0	
Chicken, boned	0	2.79	5.58	Baked	2.1	
Codfish	0	2.79	5.58	Baked	0.5	
Pork, loin	0	2.79	5.58	Baked	4.6	
Beans, green	0	2.79	5.58	Steamed	3.0	11.7% vegetables and potatoes
Corn, whole kernel	0	2.79	5.58	Steamed	1.7	
Potatoes, white	0	0.018	0.036	Steamed	7.0	
Peaches, frozen pack	0	2.79	5.58	Raw	5.9	5.9% fruits
Lard	0	0	0	Raw	10.0	10% fats and oils
Eggs, powdered whole	0	0.036	0.073	Scrambled	3.2	13.3% dairy products
Milk, evaporated	0	2.79	5.58	Raw	10.1	
Bread	0	0	0	Raw	16.0	28.0% cereal products
Flour	0	2.79	5.58	Baked	12.0	
Sugar	0	2.79	5.58	Raw	13.3	13.3% sugars, candies, etc.

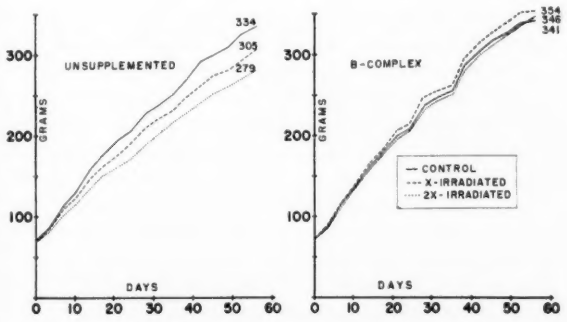

FIG. 1. Growth of male rats fed composite diets of fifteen irradiated or nonirradiated foods.

Undoubtedly, the most detailed investigation of the effects of radiation processing upon digestion of specific nutrients has been reported by Moore (32). By employing *in vitro* techniques with irradiated purified substrates and specific enzyme systems, it was found that irradiation in the absence of water did not alter the susceptibility of any of the macronutrients to enzyme action. In aqueous solution, the carbohydrates studied were partially hydrolyzed by irradiation, per se, but irradiation did not influence their further digestion by carbohydrases. The digestibility of undenatured proteins by trypsin or chymotrypsin was increased following irradiation of the protein solutions. Fats irradiated in aqueous emulsion were less well hydrolyzed by lipase than were the nonirradiated samples. This latter effect was apparently due in part to radiation-induced destabilization of the lipid emulsions.

Extension of these experiments to *in vivo* situations was also undertaken through balance studies. Three substrates (starch, corn oil and casein) were gamma-irradiated (2.79 or 5.58 megarad) in the absence of added water. These were then mixed in all possible combinations and the resultant diets fed to weanling rats. Digestion trials were made when the animals were 40 days old and again 8 months later. The most significant observation was that irradiation of starch reduced its digestibility. Furthermore, the digestibility of the irradiated starch was improved by the presence of irradiated fat in the diet. It was suggested that this may have been due to the adverse effect of irradiated fat upon gastrointestinal motility as observed by others in dogs (45). The irradiation of the lipid did not adversely affect its digestion *in vivo* after the initial short period of feeding but did decrease its utilization after prolonged feeding (8 months), the most highly irradiated sample being least well digested. However, in spite of the statistically significant differences observed in the *in vivo* experiments, Moore concludes that the changes observed were not great enough to cause any biologically significant alteration in the utilization of the irradiated macronutrients.

Radiation-induced vitamin destruction has been repeatedly reported (see references 19 and 36 for comprehensive reviews). Since the destruction of specific vitamins varies considerably from food item to food item, the interesting question arises of whether consumption of a mixed diet of many irradiated foods will overcome this

TABLE 4. *Blood Enzyme Activities in Rats Fed Composite Diets of Cooked Irradiated or Nonirradiated Foods*

Radiation Code	Serum Glutamic-Pyruvic Transaminase*			Erythrocyte Transketolase†	
	0	3	6	0	6
Supplementation					
None	24±2	19±2‡	16±2‡	1167±36	965±38‡
Thiamine	25±3	20±2‡	17±3‡	1511±49§	1404±58
Pyridoxine	23±2	26±2	25±2	1268±30	1083±47‡
Thiamine-pyridoxine				1403±48§	1366±51
B-complex	22±2	22±2	22±1		

* Per ml serum: av. ± S.E. † Per ml whole blood: av. ± S.E. ‡ Significantly lower than corresponding control at 1% probability level. § Significantly higher than unsupplemented control group at 1% probability level.

destruction. We have attempted to evaluate this point in a series of experiments performed in our Denver laboratory.

The basic diet (table 3) was formulated to approximate the military master menu for 1956 and 1957. Following irradiation all foods except the potatoes were stored at room temperature for 6 to 9 months. They were then cooked by standard army kitchen procedures before compounding the diet. It is to be noticed that two foods (bread and lard) were not irradiated inasmuch as it does not appear probable that these items will require radiation treatment for commercial purposes.

In preliminary experiments, it was shown that there was no significant difference in growth of rats fed the cooked or comparable raw diets regardless of radiation treatment. This would suggest that irradiation did not sensitize the vitamins for subsequent destruction by heat. However, growth rate decreased as radiation level increased (fig. 1). The decreased growth on the irradiated diets could be overcome by supplementation with a complete premix of the water soluble vitamins. Daily supplementation with 100 μg of thiamine did not improve the growth but daily supplementation with 180 μg of pyridoxine partially returned the growth to normal. Administration of both vitamins resulted in equal growth between control and irradiated diet groups. Brin and associates (3–5) have shown that erythrocyte transketolase and serum transaminase will serve as sensitive indices to thiamine and pyridoxine deficiency, respectively. Application of techniques developed by them (personal communication) have further demonstrated that deficiency of these two vitamins did occur in our experiments (table 4). Animals fed the unsupplemented diets exhibited decreased activities for both enzyme systems. Supplementation with the complete premix or with both thiamine and pyridoxine returned both enzyme activities to normal whereas supplementation with either thiamine or pyridoxine alone increased the activity of only the transketolase or transaminase, respectively. Thus, our experiments indicate that consumption of a mixed diet of irradiated foods, even when it contained a commercial nonirradiated bread product, resulted in a marginal deficiency in both thiamine and pyridoxine. Of these, pyridoxine deficiency would appear to be the more limiting.

SUMMARY

Chronic toxicity tests employing 22 radiation sterilized food products fed to four species of experimental animals are now in progress. Although these tests have not been completed, preliminary analysis of the data indicates that 20 of these foods are not grossly toxic to at least one species of animal (rats or dogs). Nutritional evaluation of purified macronutrients suggests that no significant biological effect will result from consumption of the irradiated products. Nevertheless, a radiation-induced improvement in the utilization of soybean protein has been reported for the rat Conversely, interesting decreases in the digestibility of irradiated starch and corn oil have also been observed. Consumption by rats of a mixed diet of many radiation processed foods causes a decrease in rat growth as radiation level increases. Oral daily supplementation with both thiamine and pyridoxine eliminates the difference in growth; supplementation with either vitamin alone provides only partial protection. When coupled with the growth data, enzymatic indices employed to evaluate the extent of vitamin deficiencies suggest that the pyridoxine deficiency may be the more limiting factor in consumption of composite irradiated diets by rats.

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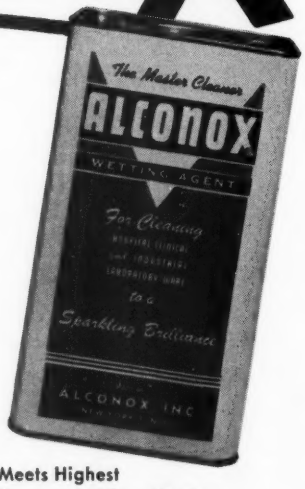

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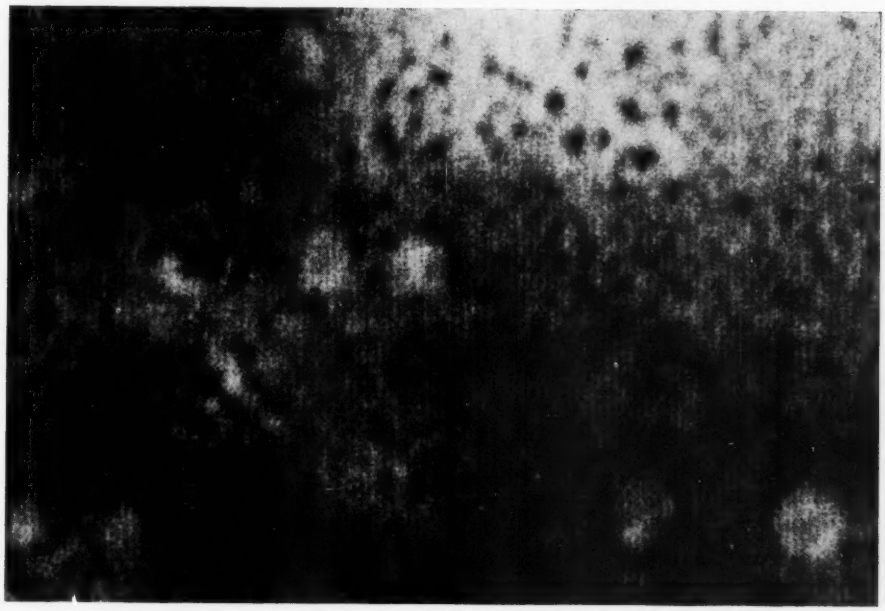

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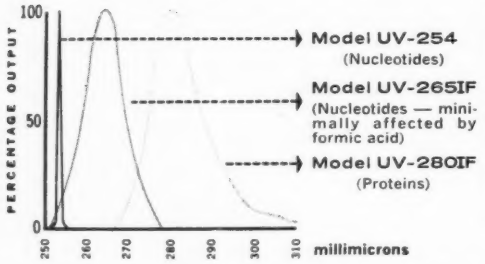

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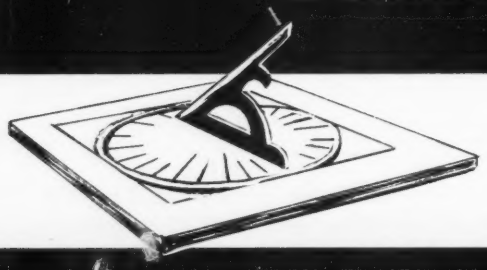

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VI. GABASE

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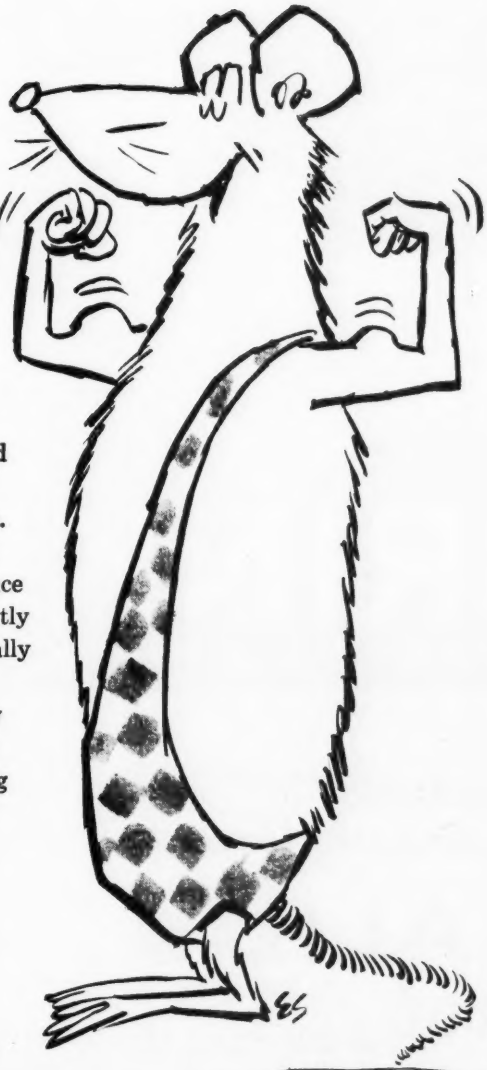

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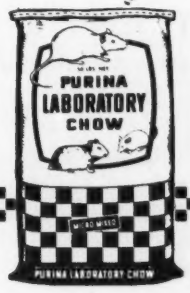

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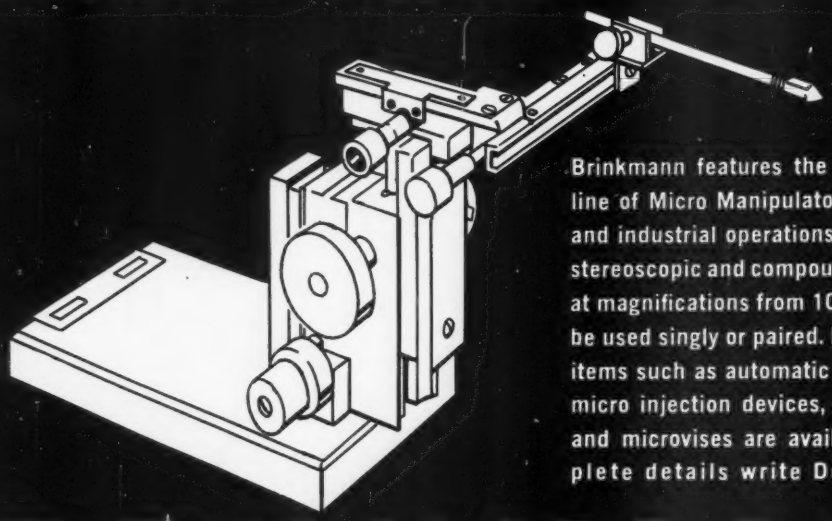

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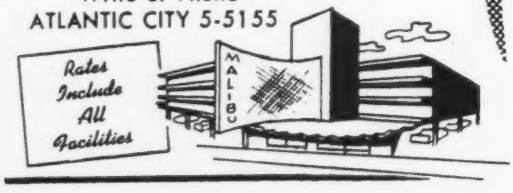

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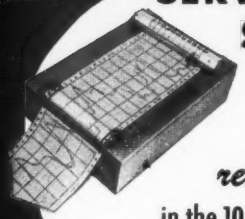

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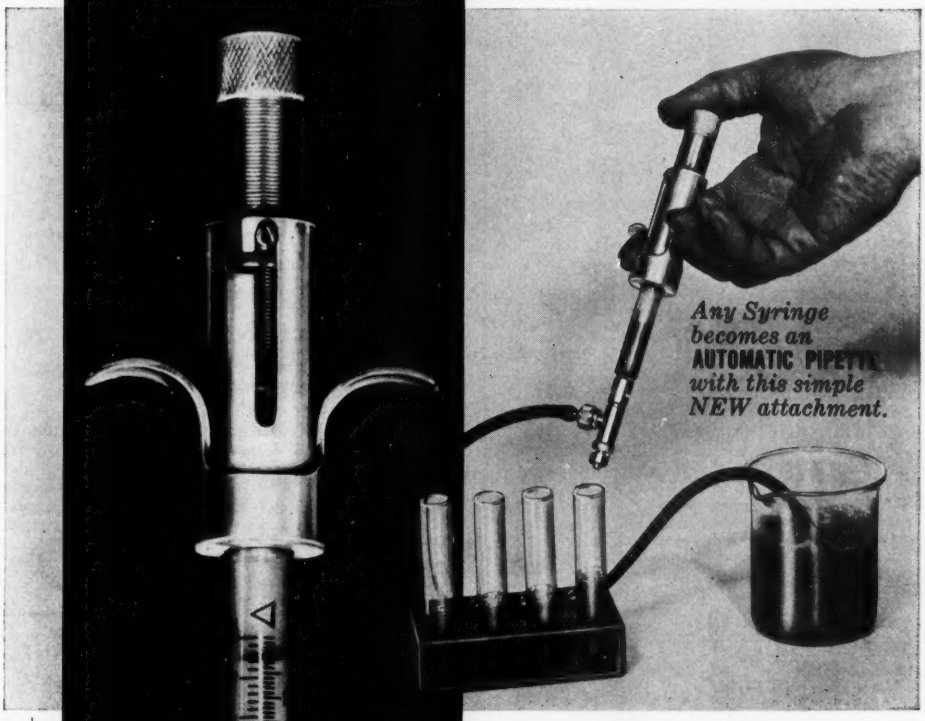

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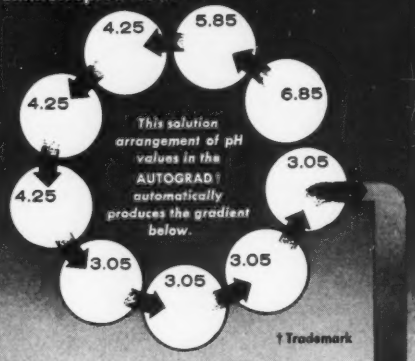

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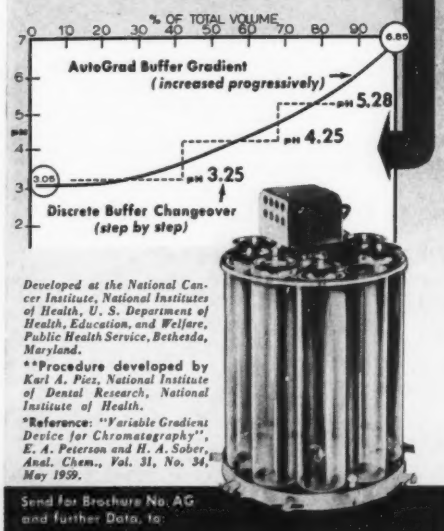

Developed at the National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Public Health Service, Bethesda, Maryland.

***Procedure developed by *Karl A. Piez, National Institute of Dental Research, National Institute of Health.*

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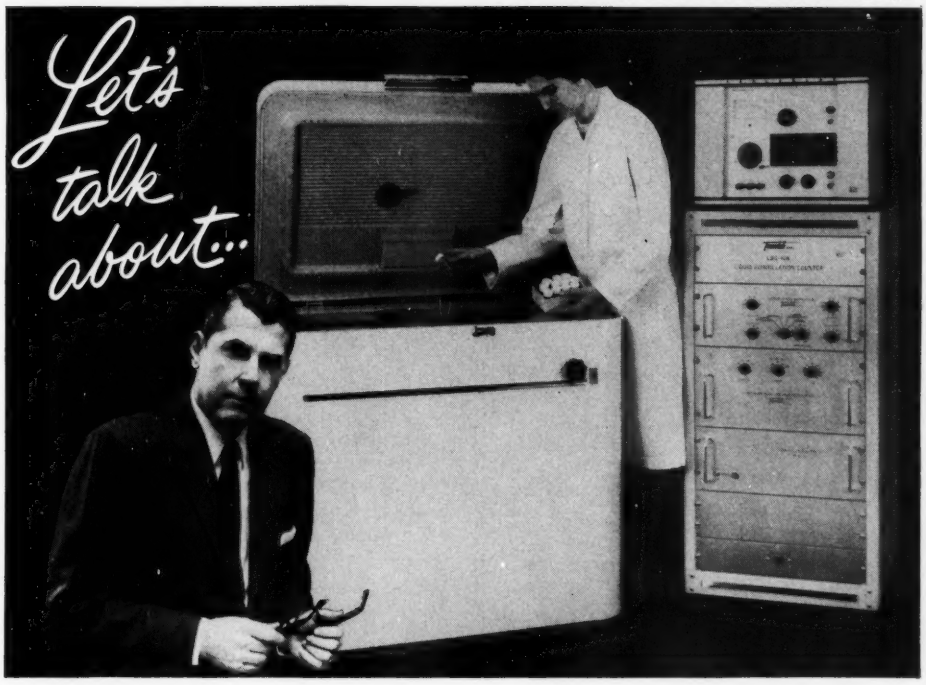

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THE ANONYMOUS MYCOBACTERIA IN HUMAN DISEASE. Edited by John S. Chapman, M.D. Springfield, Illinois: Charles C Thomas, 1960. 173 pp. $7.50

THE BASIC PHYSICS OF RADIATION THERAPY. John Selman, M.D. Springfield, Illinois: Charles C Thomas, 1960. 671 pp. $14.50

CELLULAR ASPECTS OF IMMUNITY. G. E. W. Wolstenholme and Cecilia M. O'Connor, Editors for the Ciba Foundation. Boston: Little, Brown & Company, 1960. 495 pp. $10.50

THE CHEMISTRY OF HEART FAILURE. William C. Holland and Richard L. Klein. Springfield, Illinois: Charles C Thomas, 1960. 116 pp. $5.50

CLINICAL CHEMISTRY. Second Ed. Joseph S. Annino. Boston, Massachusetts: Little, Brown & Company, 1960. 348 pp. $8.00

DYNAMIK DES EIWEISSES. Colloquim der Gelsellschaft Für Physiologische Chemis AM 9./12. April 1959 in Mosbach/Baden. Springer-Verlag, Berlin-Göttingen-Heidelberg, 1960. 200 pp. DM 29.80

ELECTRICAL STUDIES ON THE UNANESTHETIZED BRAIN. Estelle R. Ramey and Desmond S. O'Doherty, Editors. New York, N. Y.: Paul B. Hoeber, Inc., 1960. 423 pp. $6.00

FACTORS CONTROLLING ERYTHROPOIESIS. James W. Linman and Frank H. Bethell. Springfield, Illinois: Charles C Thomas, 1960. 208 pp. $8.25

PRINCIPLES OF GENERAL PHYSIOLOGY, Volume Two. L. E. Bayliss. New York, N. Y.: Longmans, Green & Co., Inc., 1960. 848 pp. $17.50

PRINCIPLES OF HUMAN GENETICS, Second Ed. Curt Stern. San Francisco, California: W. H. Freeman and Company, 1960. 753 pp. $9.50

INDEX CHEMICUS, (JOURNAL) Eugene Garfield, Editor. Philadelphia, Pa.: Institute for Scientific Information, (monthly) Rates on Request.

MECHANISMS IN RADIOBIOLOGY. Edited by Maurice Errera and Arne Forssberg. New York: Academic Press, 1960. 395 pp. $13.00

MOLECULAR STRUCTURE AND BIOLOGICAL SPECIFICITY. Edited by Linus Pauling and Harvey A. Itano. Washington, D. C.: American Institute of Biological Sciences, Second Printing 1960. 195 pp. $5.75 ($4.75 AIBS members)

PROBLEMS IN PHOTOSYNTHESIS. W. Bladergroen. Springfield, Illinois: Charles C Thomas, 1960. 198 pp. $10.50

RADIATION INJURY IN MAN. Eugene P. Cronkite and Victor P. Bond. Springfield, Ill.: Charles C Thomas, 1960. 200 pp. $6.50

SMOKING. Sir Ronald A. Fisher. Edinburgh, Scotland: Oliver & Boyd Ltd. 47 pp. 2s. 6d.

THE CHEMISTRY OF THYROID DISEASE. Rosalind Pitt-Rivers and Jamshed R. Tata. Springfield, Ill.: Charles C Thomas, 1960. 83 pp. $4.50

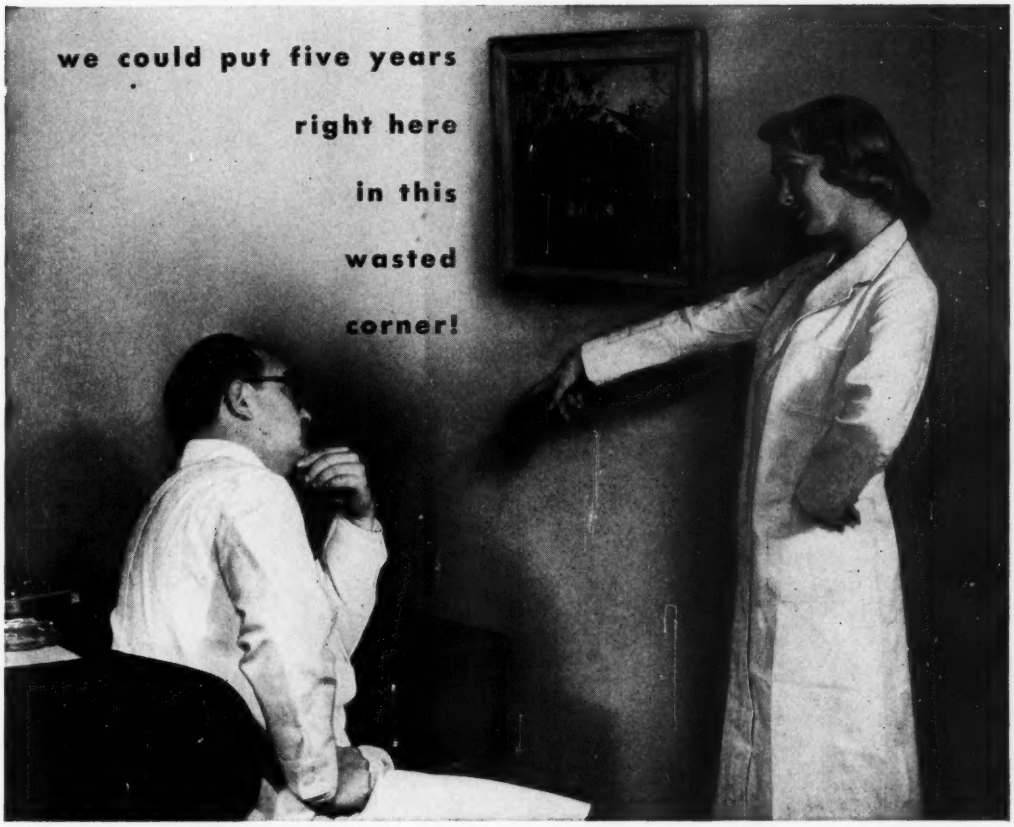
THE MECHANISM OF ACTION OF INSULIN. A symposium. Springfield, Ill.: Charles C Thomas, 1960. 320 pp. $7.50

TOWARD HEALTH AND WHOLENESS. Russell L. Dicks. New York, N. Y.: The Macmillan Company, 1960. 158 pp. $3.50

TRANSACTIONS. VOL. VI. American Society for Artificial Internal Organs. Washington, D. C.: Georgetown University Press, 1960. 380 pp. $8.00

ULTRAFILTRATION. L. Ambard and S. Trautmann. Springfield, Illinois: Charles C Thomas, 1960. 65 pp. $4.50

VISUAL FIELD DEFECTS AFTER PENETRATING MISSILE WOUNDS OF THE BRAIN. Hans-Lukas Teuber, William S. Battersby and Morris B. Bender. Cambridge, Massachusetts: Harvard University Press, 1960. 143 pp. $4.75



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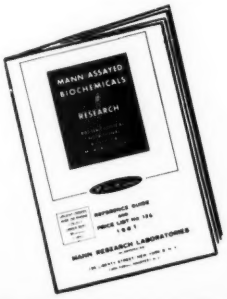

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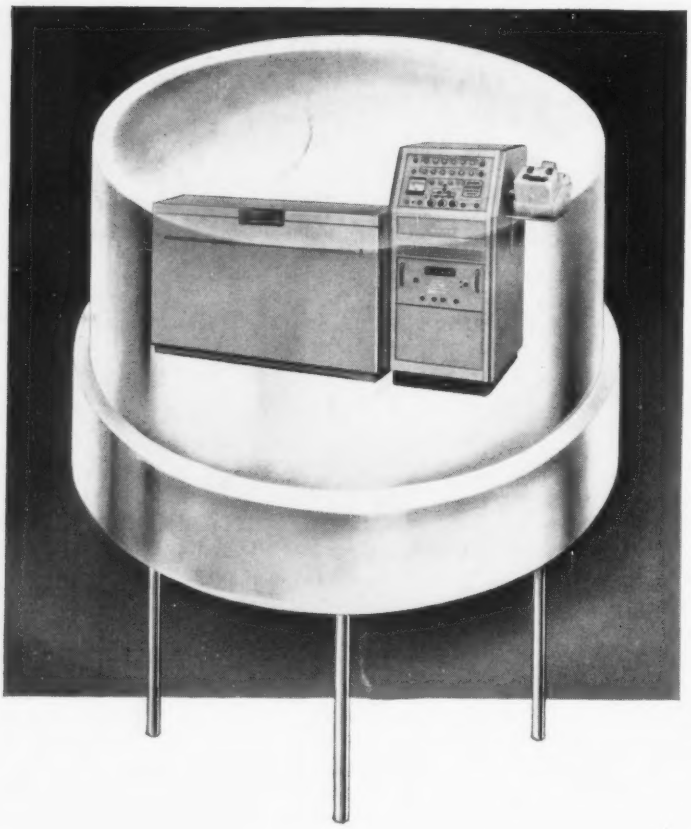

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